National Development Plan
Supporting measures for Sea Fisheries Development

Monitoring the prevalence of the parasitic dinoflagellate *Haematodinium* in Irish brown crab fisheries

FINAL REPORT

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1. General Summary

Haematodinium is a parasitic dinoflagellate that occurs in many crustacean fisheries throughout the world. Infection generally results in the death of the host species and a large component of the stock can affected at any one time. *Haematodinium* was detected in brown crab (*Cancer pagurus*) fisheries on the south coast of Ireland in winter 2003. From autumn 2004 to 2005, three geographically distinct *Cancer* fisheries were assessed for the prevalence and infection intensity of *Haematodinium.* Fisheries sampled were located in the South-East (Wexford), the South-West (Cork) and the North-West (Donegal). Macroscopic analysis was a reliable indication of late-stage infections but not of early. Late-stage infected specimens display an obvious lightening of the shell to a pale pink colour on the ventral side, with the genital opening of female crab (under abdomen) also turning from the characteristic white/clear condition to a pink/yellowish colour. At this time haemolymph appears clouded and coloured in a similar way. Macroscopically, the organs of late-stage infection specimens also turn a yellowish/pink colour and lose most of their form and shape. Haemolymph was easily extracted from samples in a non-destructive manner, and proved a relatively reliable indicator of the parasites presence when processed in the laboratory. Microscopic examination of stained blood smears revealed the presence of the parasite on all sampling occasions with the exception of autumn and winter 2004 in Donegal. Infection intensity was highest in autumn/winter months and there was a clear relationship between the prevalence of the parasite and the infection intensity in spring/summer. There was some suggestion that the parasite may pose a very significant threat to future recruitment to the fished stock, as prevalence can be very high in nursery areas. Histological preparations showed *Haematodinium* to be present in hepatopancreatic, cardiac, muscle, gill and gonad tissues at varying stages of infection, but any ‘sequence’ in the progression of infection was not determined or inferred. The study shows that *Haematodinium* may pose a significant threat to brown crab fisheries in the near future if not already doing so.

2. Synopsis of Findings

- The dinoflagellate *Haematodinium* is now (Dec 2005) prevalent in brown crab stocks throughout the three main fishing areas in Ireland (NW, SE and SW), but may not have been present in Donegal before spring 2005.
- The level of infection detected may suggest that *Haematodinium* has a significant negative impact on stock size if leading to death in all cases.
- Nursery areas show evidence of particularly high levels of infection, and this may pose the threat to future recruitment.
- Port-based sampling of catches and landings are insufficient for accurate determination of entire population infection prevalence.
- Infection level within the fished stock may not be representative of that in potential recruits, and the parasites effect on the later may pose the greatest threat.
- Microscopic examination of blood smears is a relatively effective, non-destructive method for detecting *Haematodinium*, but is time consuming and relatively expensive to process large numbers of samples.
- Externally visible symptoms in the late-stages of infection are highly reliable and easily learnt indication of infection, and should be introduced to the industry so that an effective reporting system can be developed.
- Prevalence appears to be higher in the late summer or autumn months. However, further monitoring is required to verify any inter-annual variation in seasonality.
- Infection intensity was highest in the autumn and winter months in all areas sampled.
- Spatial experiments did not detect variation in the infection intensity on a small (local) or large scale (national), but further sampling is suggested.
- There was a strong relationship between prevalence and infection intensity in the spring and summer months.
- A pilot cross-infection study may suggest that injection of infected haemolymph and consumption of infected conspecific tissue causes 100% mortality after 14days, but further experimentation is required.
- *Haematodinium* infects the hepatopancreatic, cardiac, muscle, gill and gonad tissues of *Cancer* hosts.

3. Recommendations

- Establish a routine monitoring programme for *Haematodinium* in *Cancer* and *Necora* fisheries
- Raise industrial awareness of the parasite and gross diagnostic methods by publication in the media
- Assess whether the parasite impacts/has the potential to impact other commercially valuable species
- Determine the route of infection, particularly in reference to fishing practices
- Prohibit the movement of crab derived products (particularly bait for other areas) to other geographic locations
- Conduct further research to determine appropriate management response measures to trigger when infections occur

4. Background

Brown crab (*Cancer pagurus*) represents an important and sometimes vital component of landings from many inshore potting vessels around the Irish coast. Although inshore fisheries for brown crab can be small-scale, localised and highly seasonal in some areas, fishers may still remain reliant on revenue derived from this species. The offshore vivier fleet is also 100% reliant on brown crab, which is their sole target species. Nationally, *Cancer* fisheries are only second to *Nephrops* in terms of revenue derived from crustacean species.

Haematodinium is a parasitic dinoflagellate that occurs in commercially exploited marine crustaceans (Stentiford and Shields, 2005). It has been shown previously to damage commercial stocks of Norway lobster (*Nephrops norvegicus*), snow crab (*Chionoecetes opilio*), Tanner crab (*C. bairdi*), American blue crab (*Callinectes sapidus*), and velvet swimming crab (*Necora puber*). During the numerous stages of its lifecycle the parasites affect its host in various ways but generally results in the degradation of internal organs and the blood system. This normally renders the organism unmarketable due to bad taste (hence 'bitter crab disease') and appearance, and eventually causes death. *Haematodinium* has been described as one of the most economically significant diseases of crustaceans in terms of impact on fisheries and host populations (Stentiford and Shields, 2005).

The impact of *Haematodinium* on the Irish brown crab fishery is largely unknown. In 2003, gross examination of a sample of brown crab on the south coast suggested 10-20% of individuals were in the terminal stages of infection. As external symptoms may not evident at early stages it is likely that microscopic examination of the tissue or blood would have shown that this infection rate was underestimated at the time. As information relating to the progression and spread of the disease is lacking, it is not yet possible to implement management measures to control the impact of *Haematodinium* in Irish crustacean fisheries. This was the rationale for the development and execution of the current study.

3. Objectives

- Determine the prevalence of dinoflagellate *Haematodinium* infections in Irish brown crab fisheries
- Examine the link between size/sex/condition and seasonality in levels of infection
- Where present, determine the extent of spatial variation in level of infection
- Assess impact on fisheries and recommend potential management measures

5. Materials and methods

5.1 Sampling sites and frequency

Three locations were sampled over a thirteen-month period from October 2004 to November 2005. These geographically distinct areas were located in Donegal, Cork and Wexford (Figure 1). Sampling was conducted at approximately three-month intervals, the exact periodicity being disrupted by periods when the species was not fished or was unavailable due to bad weather conditions. Table 1 shows the sampling frequency achieved at each location.

In addition to routine monitoring, an additional experiment was conducted to determine small-scale spatial variability in parasite prevalence in a randomly selected area. A vessel in Wexford was chosen, and staff from TCD/GMIT sampled haemolymph (using the methodology described below) from the first 15 individuals being cleared from each of eight consecutively hauled fishing strings of 25pots each.

Figure 1. Map of Ireland showing the three locations where brown crab fisheries were sub-sampled for *Haematodinium*

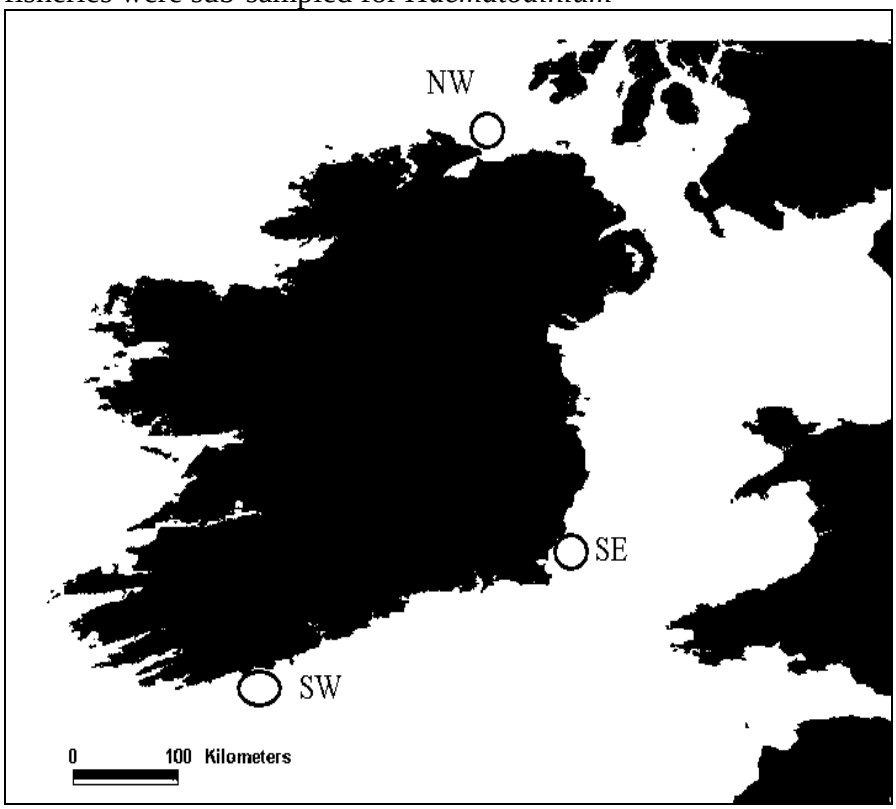


Table 1. Date and number of crabs sub-sampled for each location from November 2004-2005

Location	Date	Number
Donegal	November 2004	48
	April 2005	59
	May 2005	50
	August 2005	50
	November 2005	50
Cork	November 2004	50
	June 2005	50
	August 2005	49
	October 2005	50
Wexford	November 2004 (early)	45
	November 2004 (late)	169
	December 2004	50
	March 2005	47
	June 2005	89
	August 2005	60
	November 2005	50

The samples supplied by index vessels appeared to suggest that smaller crabs tended to be more susceptible to *Haematodinium* infection. Increasing concerns from fishermen over the number of infected juvenile individuals in Donegal at the end of

November 2005 prompted a final experiment to assess infection level on nursery grounds. As the end of this project was approaching it was not possible to conduct microscopic analysis of large numbers of individuals due to the high labour input required, but an external assessment of 186 female crabs with a carapace width <140mm was conducted on a known nursery area. The examinations were conducted by a senior member of GMIT staff with experience in verifying infection. The shell was scrutinised and the blood of all individuals was examined for clouding by removing a leg and placing haemolymph onto a sheet of white paper. The carapace of every 5th crab was removed to verify diagnosis by examination of the condition of the internal organs. Although this method was assumed to be 100% effective in identifying late-stage infections, it should be appreciated that other apparently healthy individuals may have been in an early stage of infection.

5.2 Tissue samples

On each sampling occasion crab haemolymph samples were collected for microscopic examination. Approximately 50 inter-moult specimens were randomly selected from commercial **catches** from index vessels. Participants were requested to land several boxes of animals representative of the **catch** as apposed to the **landings**. This allowed all components of the stock to be monitored, although soft-shelled crabs were not sampled. It should be appreciated that in some areas undersized crab are uncommon, and in these instances sampling may not take account of the whole stock (effectively representing only landings).

Carapace width (CW) of each crab was measured (mm) and total weight (g) recorded. All individuals were also sexed and examined for external symptoms of infection. A 1ml syringe with a 19-gauge needle was used to extract 0.5 ml of blood from between the 5th periopod and the main body cavity or from the joint between the first periopod and the carapace. This sample was injected directly in to a labelled 'Vacutube' containing 0.5ml of 4% formalsaline to create a 1:1 mixture. Repeated daily smearing of blood samples indicated that they could be stored for up a week at 4°C before slide preparation and remain viable, but were processed within 24hr after this trial was conducted.

5.3 Validating external diagnostics

Forty *Cancer pagurus* were taken from the landings of a small inshore crab fishing vessel based in Slade Harbour, Co. Wexford during winter 2004. Fifteen individuals were non-randomly selected from the catch on the basis of outward symptoms of *Haematodinium* infection and fifteen more for their seemingly healthy condition. Infection symptoms consisted of pink areas of shell where the leg joints met the body and under the flap of the abdomen. Further collection was made from the catches of a small in-shore fishing vessel based in Baltimore, Co. Cork. Five infected crabs and five uninfected crabs were non-randomly selected. Additionally, on the first 2 sampling occasions at each location, the primary scientist extracting blood made an assessment of whether they considered the specimen infected or not based on external appearance and haemolymph colour.

All collected crabs were transferred live to the laboratory in cooled containers. Dissection and haemolymph extraction was conducted immediately after specimens were humanely killed by chilling and 'pithing'. Hepatopancreas, gonad, cardiac muscle and claw muscle samples were removed and fixed in chilled 10% buffered formalsaline.

5.4 Tissue preparation and analysis

As stated above blood smears were prepared for each individual within 24hr of sampling. These were stained using Giemsa stain. This staining technique is well established for the identification of *Haematodinium* in other crustaceans (Stentiford and Shields 2005). After preparation and staining, blood smears are fixed and can be stored/re-analysed indefinitely.

Blood smears were viewed at x400 magnification using a compound microscope. All smears were read on multiple occasions by several trained readers to ensure accurate results. Each slide was scanned for the signs of infection. The vegetative state of the parasite was used as the main indicator of infection, this is characterised by distinctive cells containing one or more nuclei of dinokaryon nature. These uninucleate and multinucleate forms are from the vermiform filamentous trophont stage of the life cycle (Field and Appleton, 1992), and are by far the most commonly observed.

The overall prevalence of *Haematodinium* infection was identified as the number parasite cells as a proportion of the total cell number examined. To quantify levels of infection, 300 cells were counted which included all types of bloods cell and uninucleate and multinucleate *Haematodinium* cells. Three intensity calculations were carried out for each individual crab to determine the levels of infection. These were:

1. Overall intensity = ((u + m) / t) x 100
2. Uninucleate intensity = (u / t) x 100
3. Multinucleate intensity = (m / t) x 100

Where u = number of uninucleate cells, m = number of multinucleate cells and t = total number of cells.

5.5 Embedding

Fixed tissue samples were prepared for embedding in paraffin wax to facilitate examination using light microscopy. A piece of tissue approximately 5mm^3 was separated from each fixed sample and placed in a plastic capped glass bottle with a paper label for the remaining procedures. The tissue was dehydrated *via* sequential submergence in alcohols of decreasing dilutions (70%, 90%, and two rounds of 100%). Each wash lasted 1hr and excess liquid was decanted into a waste receptacle before the introduction of any new solution. The tissue was cleared for 1hr in the synthetic solvent 'Histoclear' when completely dehydrated. A Histoclear/paraffin wax "mush" (a roughly 1:1 mixture) was then poured in with each sample and heated for an hour at 60°C. Each sample was drained of this mixture and then covered with pure paraffin wax. To ensure full infiltration of the tissue and to promote softening of any tissue hardened in the fixation process, the samples were left in this wax over night in an oven at 60°C. The wax was drained and the sample covered by fresh wax the following day. This was left for at least an hour in an oven at 60°C. A small amount of tissue was taken from each sample and placed in a plastic mold to prepare for microtome sectioning. New wax was poured into the mold, and a labelled plastic cover was pressed onto the wax to produce a uniform shape. The blocks were then placed on a cold plate and left overnight to harden. Specific orientation was deemed unimportant for most tissue types; however, the gill tissue was always placed with the folds facing outward so that the lamellae could be viewed after sectioning.

5.6 Sectioning

A standard steel-blade rotary microtome was used to section the embedded tissue to a width of 8–12µm. A series of three or four slices were floated onto the surface of a beaker of warm water to smooth any wrinkles resulting from sectioning. A few drops of an egg white–thymol mixture served as an adhesive to attach these sections to standard glass slides. Frosted slides were labelled and dunked into the water under the section which were swept onto the slide as it was removed from the water. The slides were left to air dry.

5.7 Staining

Slides were stained using haemotoxylin and eosin (H&E). Haemotoxylin acts as a stain for acidic components of cells, including the chromatin and nucleoproteins, colouring them varying shades of blue. The eosin is acidophilic staining the basic components of tissues and parasite cells varying shades of pink. The slides, placed in metal racks in sets of 10–12, were first de-waxed using ‘Histoclear’. Because H&E is water-based, all slide samples were hydrated in a series of 3min baths in 100% (twice), 90%, and 70% ethanol (EtOH), followed by a rinse in distilled water to remove all excess ethanol.

The dehydrated slides were immediately immersed in a prepared solution of haemotoxylin for 5min and then rinsed briefly to remove excess stain. A 15-30sec rinse in Scott’s Tap Water served to differentiate tissue. Slides were then counter-stained in a previously prepared bath of eosin for 4min. Slides were rinsed for any excess stain and dehydrated in an opposing sequence of immersions to that outlined for hydration above. Once returned to ‘Histoclear’ slides were mounted placing them face-down on a cover slip covered with a few drops of the mounting agent ‘DPX’. A small amount of pressure was applied to ensure the mounting agent spread across the entire cover slip.

5.8 Qualitative Tissue Analysis

A subjective approach was taken to separate the tissues into categories based on the quantity and spread of parasite cells and the extent of tissue damage. A protocol was developed to separate the tissues into five stages of infection severity, zero representing no infection, through to four representing very high infection.

Stage Zero – No infection.

Stage One: Only one or very few parasite cells. Cells found only within connective tissue. No tissue damage related to infection noted.

Stage Two: Fairly wide-spread with light coverage of parasite throughout connective tissue. Tissue damage generally contained to connective tissue with visible infection only in cardiac and gill tissues. Although gill tissue contains the parasite, it does not appear excessively damaged. Gonad tissue, muscle tissue, hepatopancreas tubules free of parasite cells.

Stage Three: Thick masses of parasite cells but with some large tracts of tissue remaining. Some light damage to gill tissue but more large scale damage in other tissues. Most muscle tissue not invaded but surrounded by parasites. Hepatopancreas tubules with one or two parasite cells present within the lumen.

Stage Four: Thick masses of parasite cells in most or all of the tissue types. Parasites throughout all parts of tissue including muscle and within lumen of hepatopancreas tubules.

5.9 Cross-infection experiment

The route by which *Haematodinium* infects a host is poorly understood, and may be host species-specific. Fishermen involved in this project perceived there to be 2 instances where infection of crab appeared to be more serious:

1) Areas where spring/summer whelk fisheries are established on autumn/winter crab grounds

2) Where a high degree of clawing activity had occurred for a number of years previously

The research group considered that both of these conditions shared a commonality in that they both had the potential to introduce/re-introduce weak, dead and dying individuals that may represent a source of food for scavenging conspecifics. In the former case infected individuals may be shipped in from other areas, while in the later the survival of an infected animal may be further compromised by removing the claws

and discarding the body. It is possible that in both cases direct consumption of infected tissue may not be the primary vector, and that sporilation from dead and dying individuals raises the numbers of parasites in the surrounding habitat. In order to determine the likely importance of these potential vectors, forty healthy female intermoult crab between 140-150mmCW were transported from Donegal to Wexford in December 2004, when there was no infection detected in the former area. Blood samples were taken to ensure all specimens were free from *Haematodinium* infection. The following treatments were then arranged:

1) Ten individuals were randomly selected and injected with haemolymph drawn from an infected (confirmed by haemolymph examination) Wexford 'donor' crab.

2) The infected muscle of the donor was fed to ten further individuals that readily ate when submerged in a seawater filled tank.

3) A further ten individuals were placed in another tank containing the remainder of the carcass of the donor for 30min, but were prevented access to this food source. This treatment simulated external contact with the parasite cells, but it should be appreciated that due to lack of knowledge relating to the lifecycle, these may have not been capable of invading the potential hosts.

4) The remaining individuals were not exposed to *haematodinium* in any way.

All individuals were placed into a large holding drum and attached to a buoy in a sheltered bay in a sub-tidal zone. Blood samples were extracted from surviving animals after 14days. The internal organs of dead individuals were examined.

6. Results

6.1 External macroscopic diagnosis

External symptoms of infection in the fisheries sampled were identical for each area. There was a pinkish lightening of the shell on the ventral surface (blue arrow Figure 2) and of the joints (red arrow Figure 2) in both males and females. A pinkish lightening of the opening to female oviducts (red circle Figure 2) and pleopods (Figure 3) was also very evident.

Figure 2. Comparison of late-stage *Haematodinium* infected (top) and healthy (bottom) female *Cancer pagurus* from Wexford, winter 2004. Abdominal flaps removed in both cases.

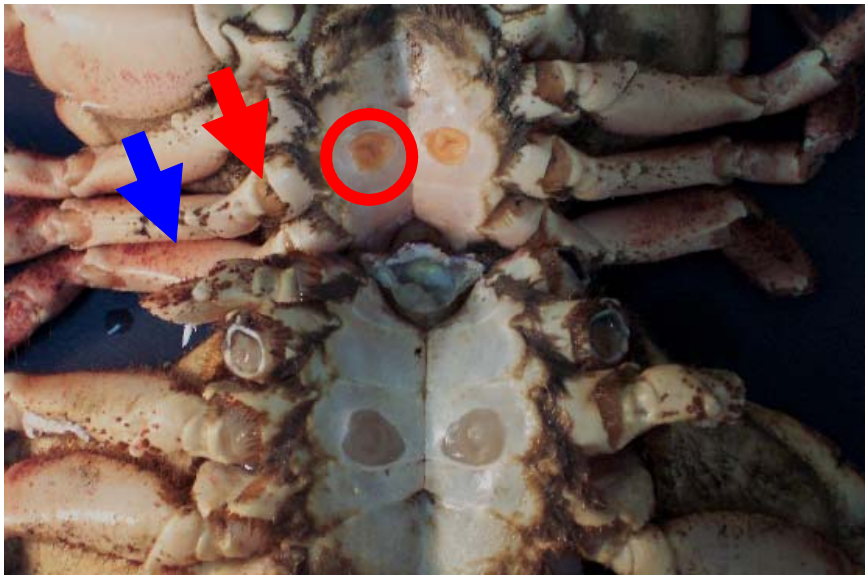


Figure 3. Comparison of pleopods from late-stage *Haematodinium* infected (left) and healthy (right) female *Cancer pagurus* from Wexford, winter 2004.

6.2 Internal macroscopic diagnosis

The haemolymph of late-stage infected individuals was cloudy in appearance, Figure 4, when compared to the healthy clear condition. Removal of the protective shell surrounding the claw muscle also showed discolouration associated with the late-stages of infection (Figure 5). In the very late stages of infection, the internal organs of the host were not distinguishable having turned light yellow and losing all shape or form. Figure 6 displays how easily the late-stage infection animals can be identified when compared to healthy individuals by removal/breaking open of the carapace. The close up picture of the muscle tissue surrounding the heart shown in Figure 7 is a clear indication of the level of tissue degradation present immediately prior to death.

Figure 4. Comparison of haemolymph from late-stage *Haematodinium* infected (right) and healthy (left-in red circle) female *Cancer pagurus* from Wexford, winter 2004.

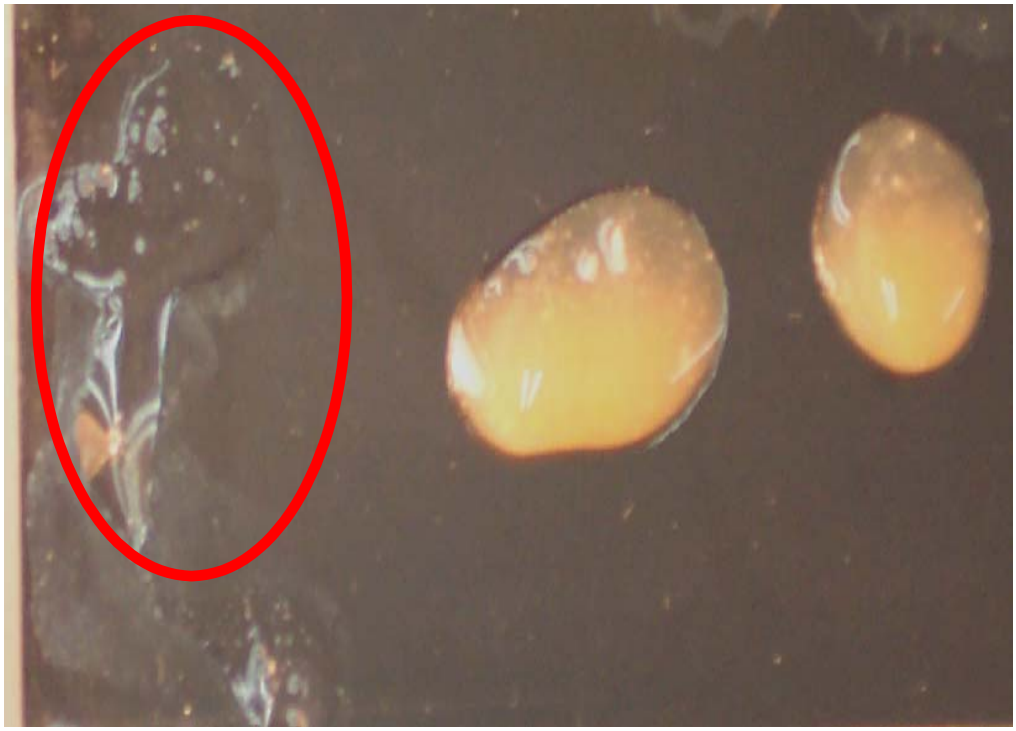
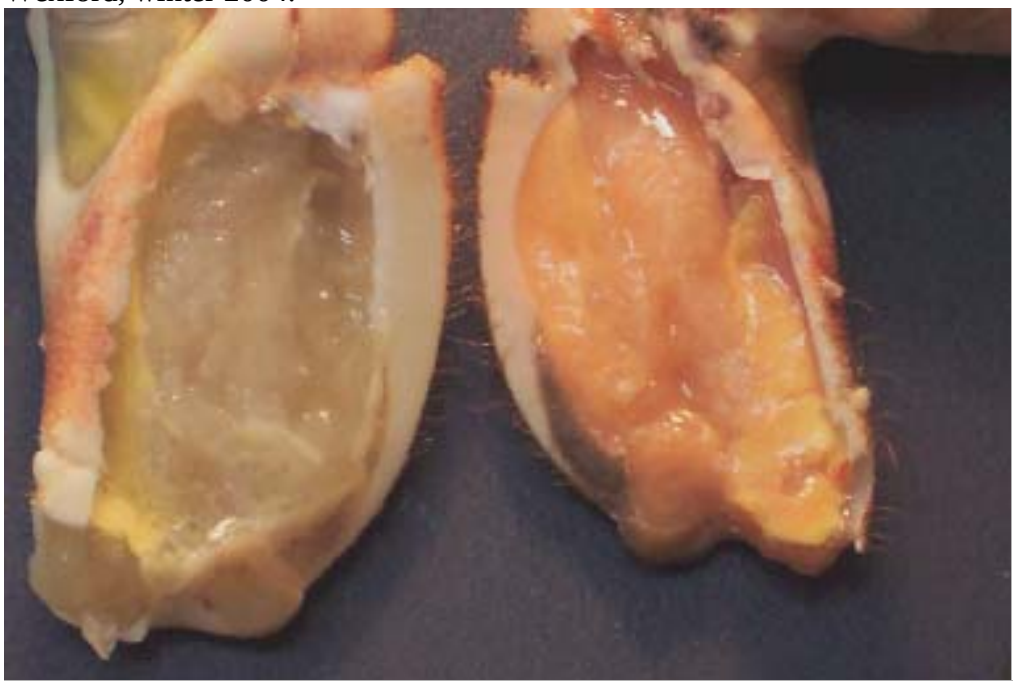


Figure 5. Comparison of claw muscle (shell removed) from late-stage *Haematodinium* infected (right) and healthy (left) female *Cancer pagurus* from Wexford, winter 2004.



6.3 Validation of external symptoms as a diagnostic

All of the individuals that were suggested as being infected during port sampling on the first 2 visits to each location were subsequently confirmed as carrying the parasite. On several occasions however, individuals that had appeared healthy were subsequently diagnosed as hosting light infections. External symptoms therefore gave a 100% reliable confirmation of an individual being infected, but only an 85% reliable indicator when applied over an entire sample.

The samples taken from Cork were correctly identified as infected and uninfected individuals, but sample numbers were low (5 of each) due to poor survival in transit.

Of the 40 individuals taken from Wexford (15 with external symptoms, 15 healthy), external symptoms were again a 100% reliable indicator of infection of an individual. The parasite was found in the blood and histological preparations of other organs (hepatopancreas, gonad, cardiac muscle and claw muscle) in all individuals showing external symptoms.

Interestingly, although the parasite was not found in the blood of the 15 ‘healthy’ specimens processed, 3 of these individuals showed low levels of infection in other organs. A single parasite cell was observed in one of all five tissue slide preparations for one of these individuals, while stage one infections were observed in most tissue types for the other two. This may suggest that invasion of the blood and the rapid parasite cell multiplication observed in badly affected individuals may only occur in the late stages of infection and/or another organ provides an initial entry point. There is much speculation as to the route *Haematodinium* takes to enter the host, and this may be species-specific. Considering the results of this single experiment may suggest that blood smears are only 83% reliable as a diagnostic for the parasite in *Cancer pagurus* but repartition is suggested.

Figure 6. Comparison of internal organs (carapace removed) from late-stage *Haematodinium* infected (bottom) and healthy (top) female *Cancer pagurus* from Wexford, winter 2004.

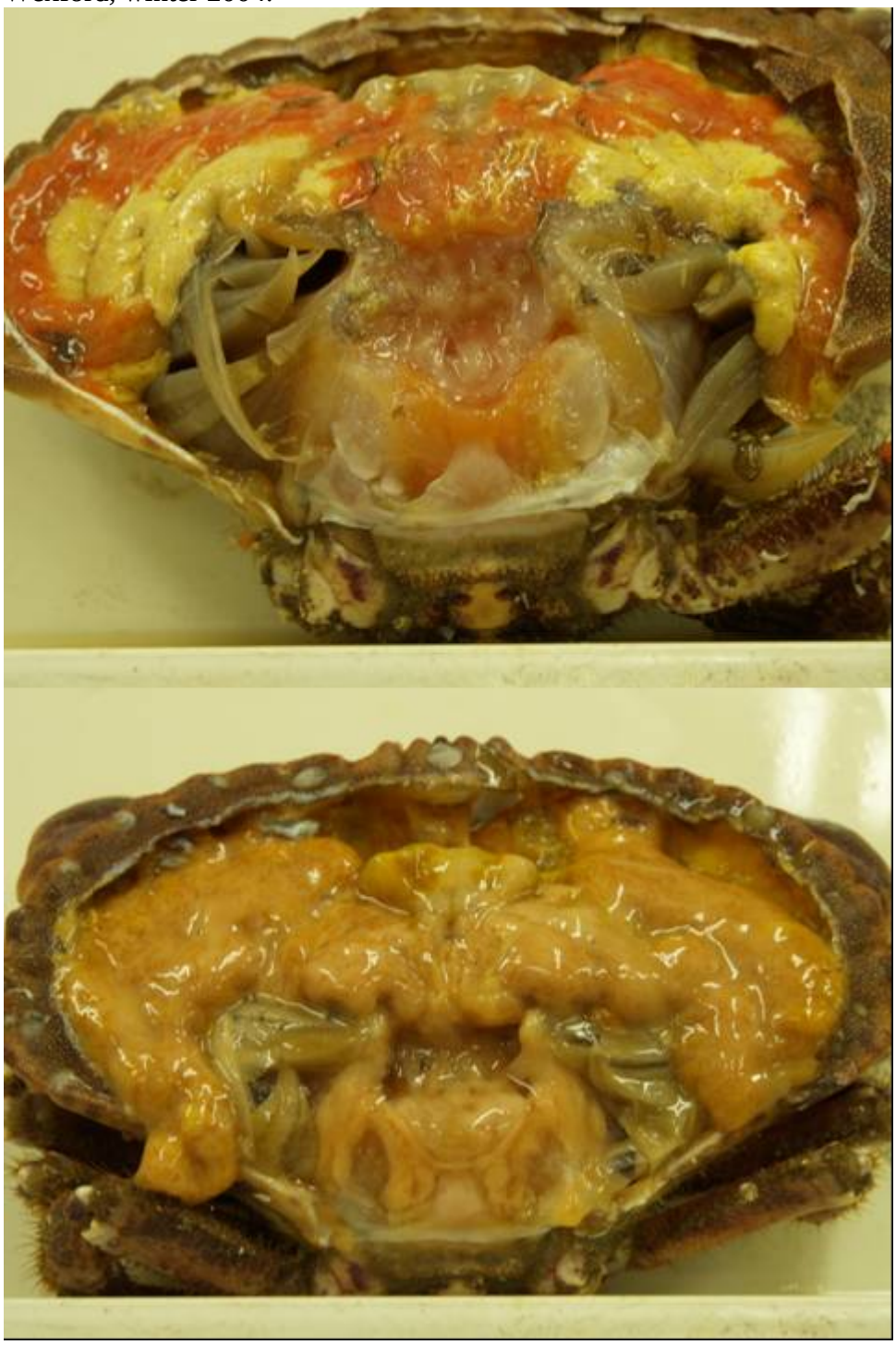
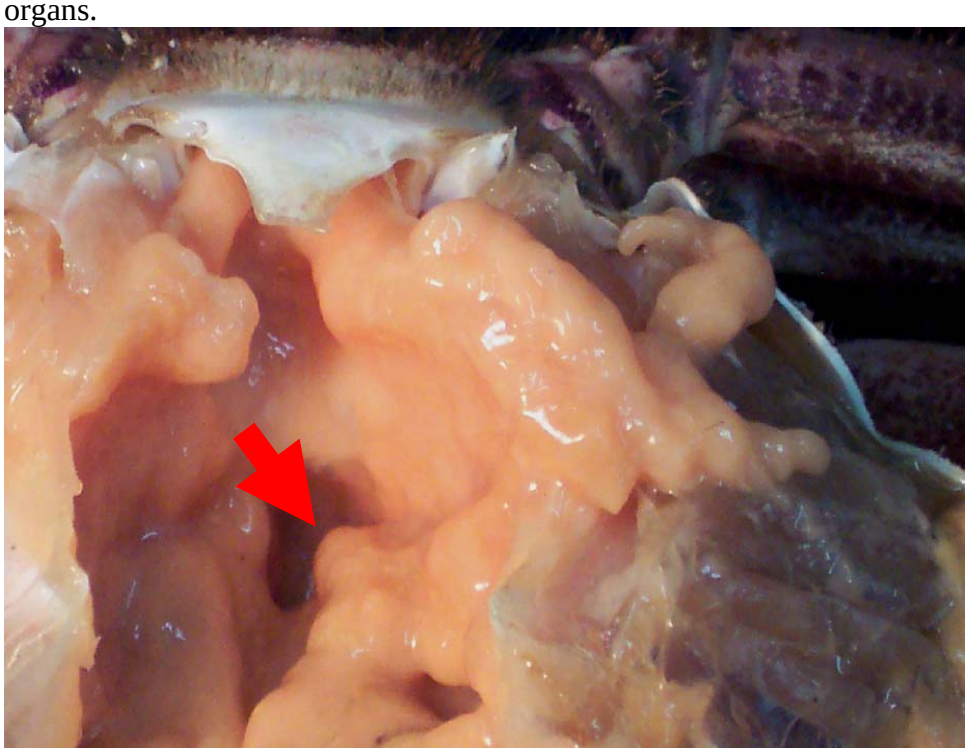


Figure 7. Close-up of tissue surrounding heart cavity (carapace and heart removed) showing massive tissue degradation caused at late-stage of infection. The ovaries of this female remain *in-situ* (red arrow), but are barely distinguishable from other organs.



6.4 Haemolymph microscopic diagnosis

Normal crustacean haemolymph contains 3 types of cell, hyalinocytes, semi-granulocytes and granulocytes (Figure 8). The exact functions of each of these cells are not fully described, but in general increases in the later two cell types are associated with host immune response. Microscopic examination of blood smears conducted in the current study clearly showed varying stages of infection intensity in the haemolymph. Figure 9 shows a light early-stage infection where only a few parasite cells could be seen. At this stage of infection the compliment of host cells appeared unaffected. In the late-stages of infection, Figure 10, the parasite filled much of the available space in the haemolymph and was clearly seen to be actively dividing. Unbound chromatids and multi-nucleate cells were visible in high numbers, Figure 11. At this late stage of infection few of the hosts blood cells remained and consisted mainly of semi-granulocytes and granulocytes.

Figure 8. Blood smear from healthy *Cancer pagurus* showing 1) Hyalinocytes; 2) Semigranulocytes; 3) Granulocytes.

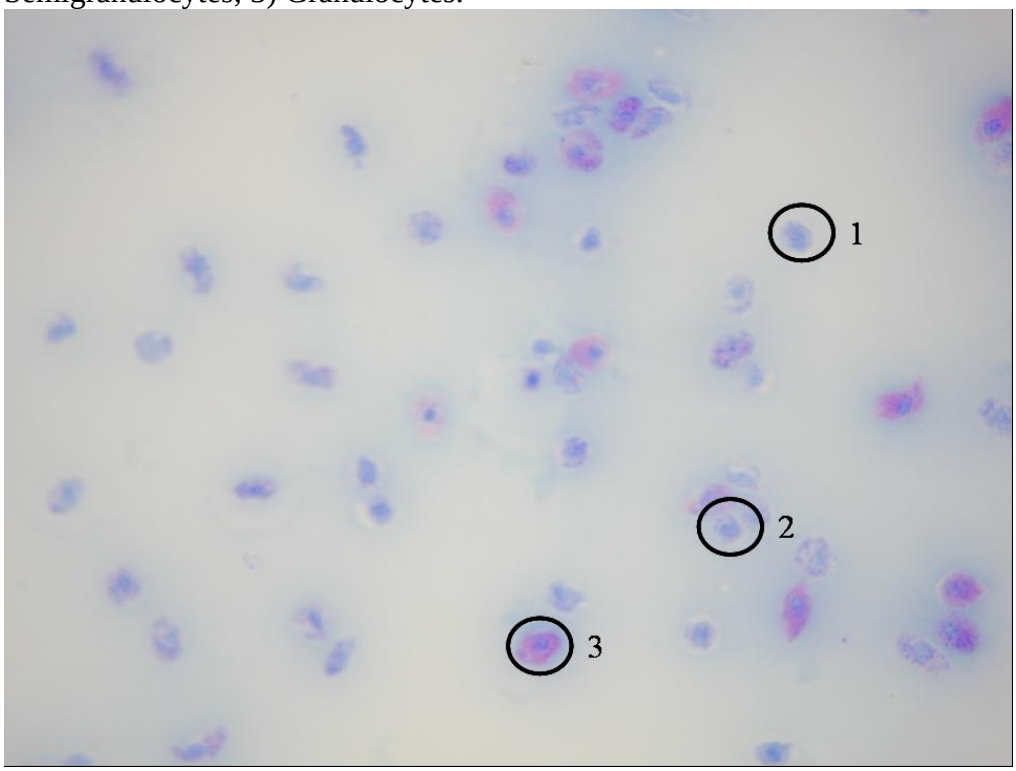


Figure 9. Blood smear of *Cancer pagurus* showing light infection of *Haematodinium* showing 1) Parasite cells with large cytoplasm and highly condensed nucleus; 2) Bi-nucleate parasite cells

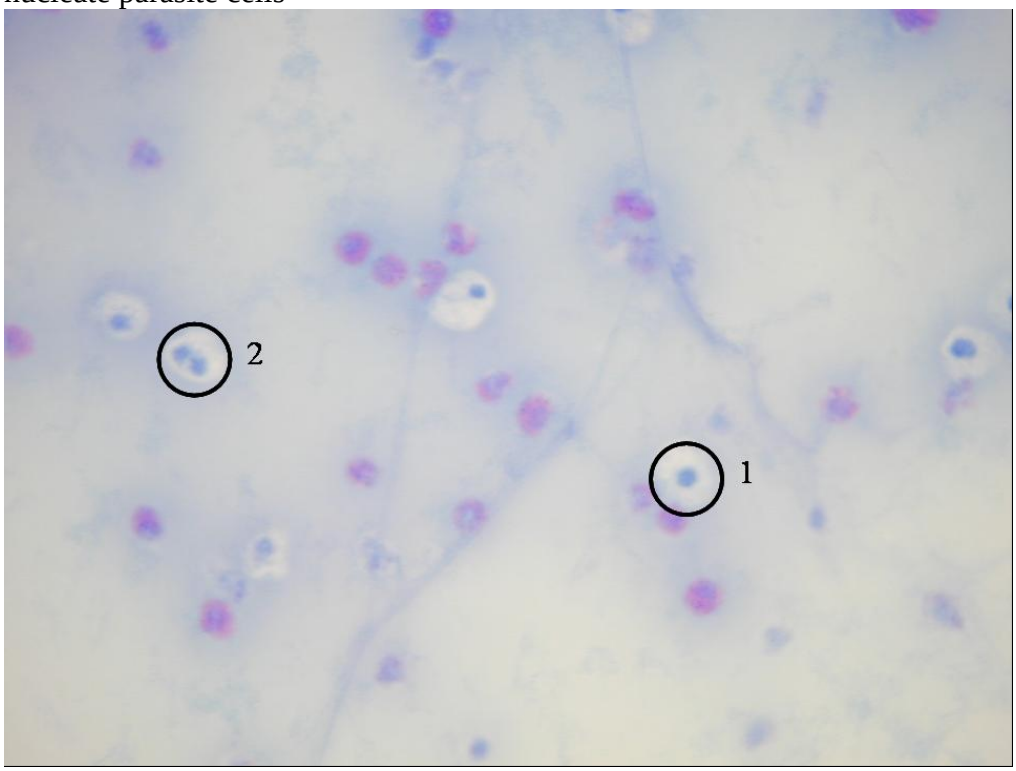


Figure 10. Blood smear of *Cancer pagurus* showing heavy infection of *Haematodinium* showing uni-, bi- and multi-nucleate parasite cells, many with unbound chromatids which evidence very rapid cell multiplication. Few host cells remain.

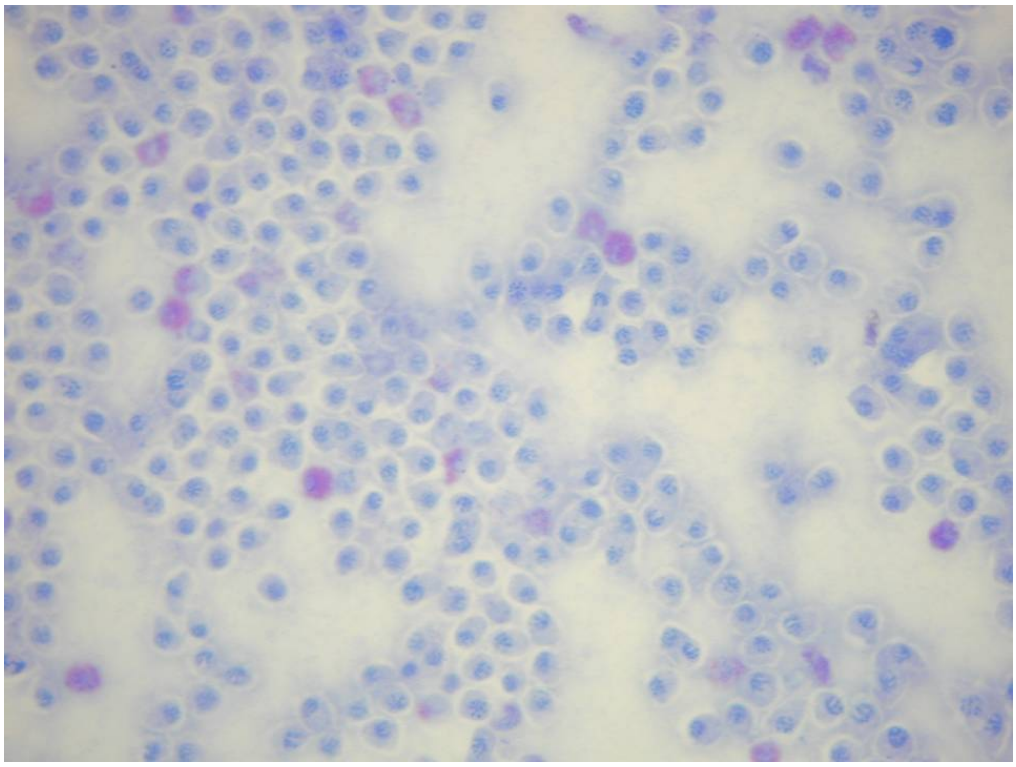
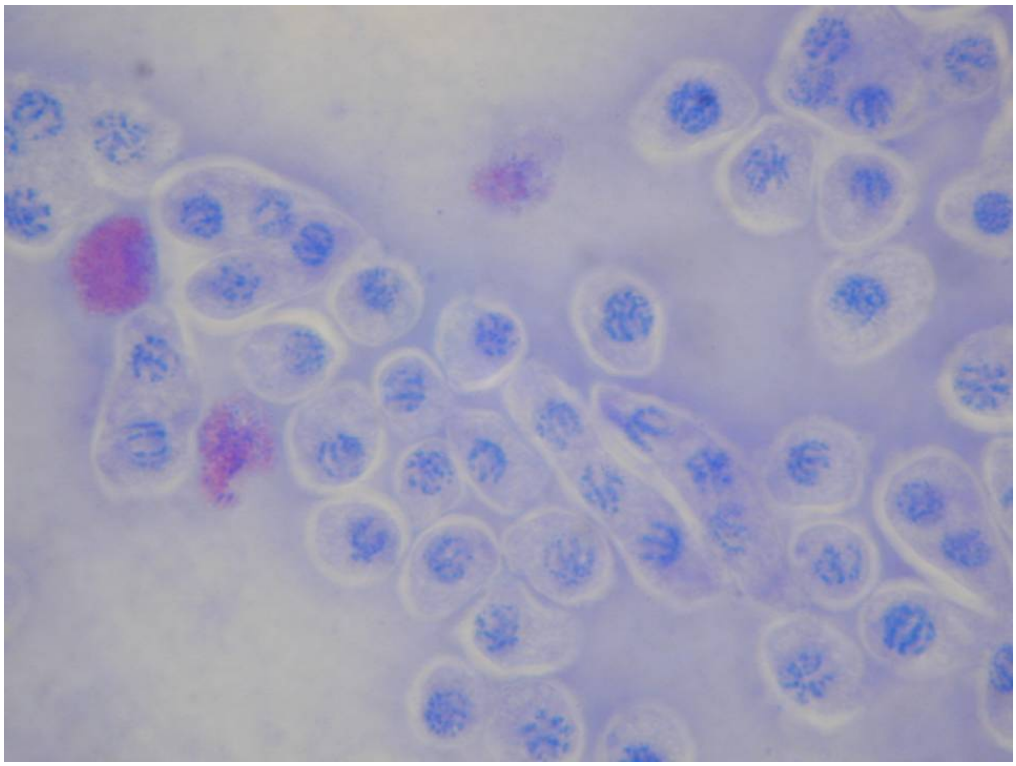


Figure 11. Close up of blood smear of *Cancer pagurus* showing heavy infection of *Haematodinium*.



6.5 Size, weight and sex ratio

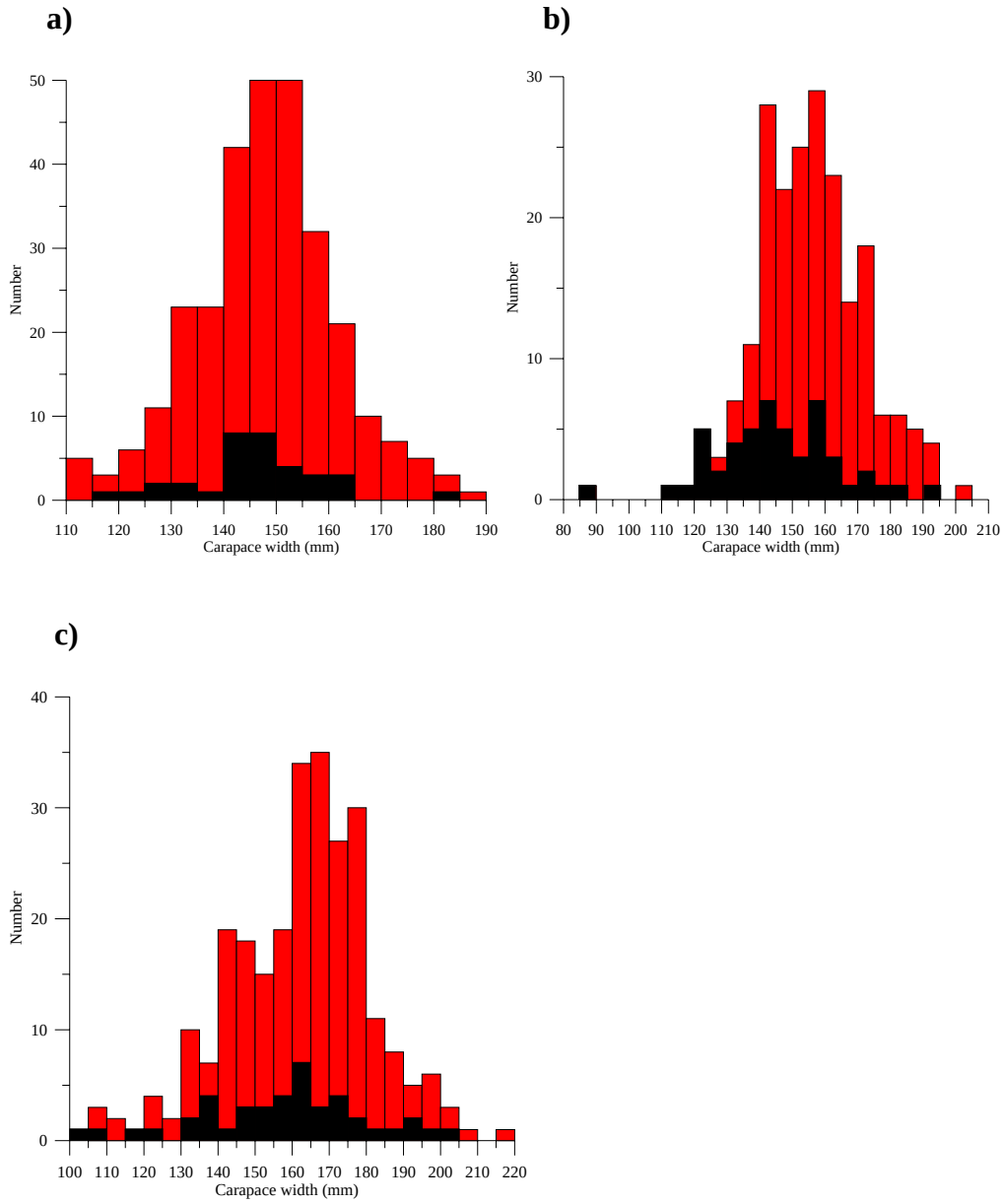
During the course of this project 757 crabs were sampled of which 677 were female and 80 were male (M:F sex ratio of 0.1:1). Mean carapace width sampled on any one day ranged from 134.8±15.9mm to 176±14.8mm (Table 2). Lowest (381.2±135g) and highest (813.6±207g) total mean weight was associated with the same two dates respectively (Table 2). The variation in the size and weight of individuals sampled on any one day was dependent on availability. Although a fish box full of individuals of a size range and sex ratio representative of the **catch** was requested this differed both between- and within-areas temporally. Due to the highly skewed sex ratio, males could not be sampled on all occasions. There was no significant difference between the weight of infected and uninfected specimens.

Table 2. Mean size, weight and sex of the brown crab sampled in Wexford, Cork and Donegal in 2004 and 2005.

		N	Female	Male	Mean Carapace width (mm)	±SD	Mean total Weight (g)	±SD
Wexford	Nov-04	45	45	0	148.3	9.7	478.2	101
	Dec-04	50	50	0	153.5	13.8	567.1	168
	Mar-05	47	25	22	134.8	15.9	381.2	135
	Jun-05	50	29	21	148.3	13.5	510.0	132
	Aug-05	50	48	2	150.9	7.4	503.4	66
	Nov-05	50	50	0	154.4	9.4	564.4	102
Cork	Nov-04	59	54	5	158.6	12.3	589.9	157
	Jun-05	50	45	5	156.3	16.8	593.0	223
	Aug-05	49	46	3	160.8	13.6	616.1	159
	Oct-05	50	43	7	146.1	19.8	501.3	222
Donegal	Nov-04	48	48	0	172.1	6.5	763.7	81
	Apr-05	59	59	0	147.4	14.7	495.6	158
	May-05	50	50	0	176.0	14.8	813.6	207
	Aug-05	50	50	0	164.5	11.6	647.1	126
	Nov-05	50	35	15	159.0	24.6	641.9	301

There was no significant difference in the size-frequency distribution (carapace width) of individuals infected in each location, Figure 12, when compared over the entire year. Individuals of a carapace width <130mm were poorly represented in samples, as these were not common in many fishing areas.

Figure 12. Pooled size-frequency (carapace width mm) distributions for *Cancer pagurus* sampled in each location a) Wexford, b) Cork, c) Donegal November 2004-05. Sexes pooled, black bars show positive for *Haematodinium* infection (microscopic determination-see methods).



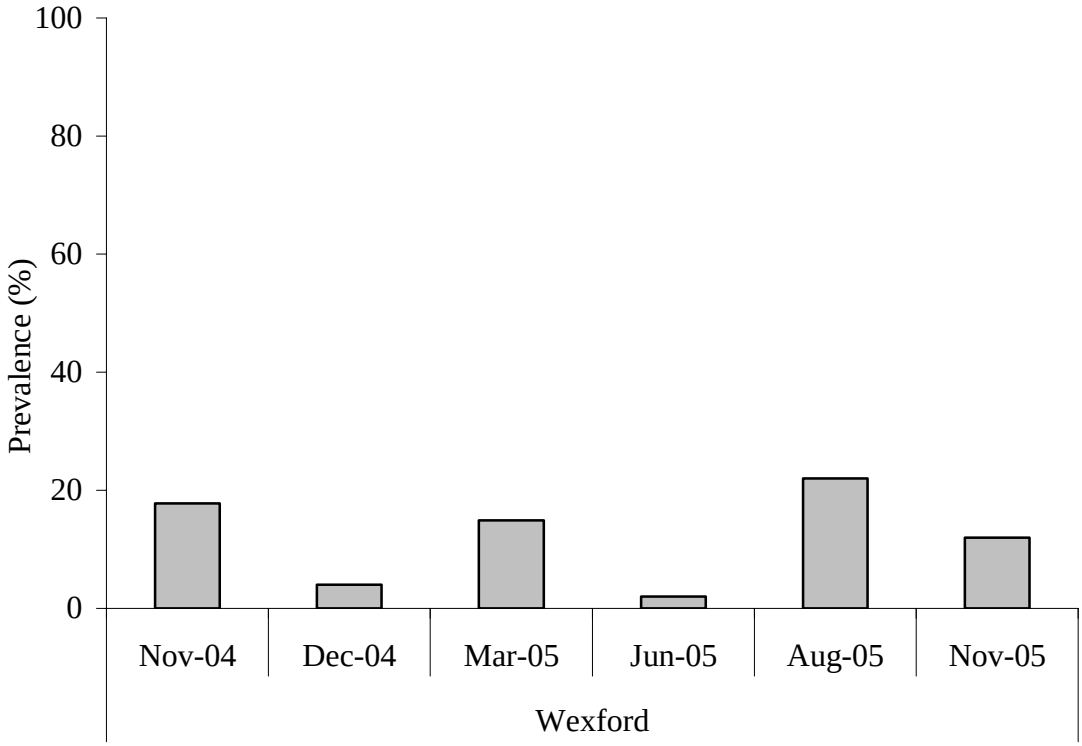
6.6 Prevalence

The prevalence of *Haematodinium* in sub-samples (expressed as the percentage of infected individuals) from 2004/2005 was calculated for Wexford, Cork and Donegal. The parasite was encountered at all sites on all occasions with the exception of Donegal in November 2004.

6.6.1 Wexford

In Wexford lowest infection levels were recorded in December 2004 and June 2005 with prevalence of 4% and 2% respectively, Figure 13. Prevalence levels >15% were recorded on 3 occasions in November 2004, March 2005 and August 2005 (18, 15 and 22% respectively).

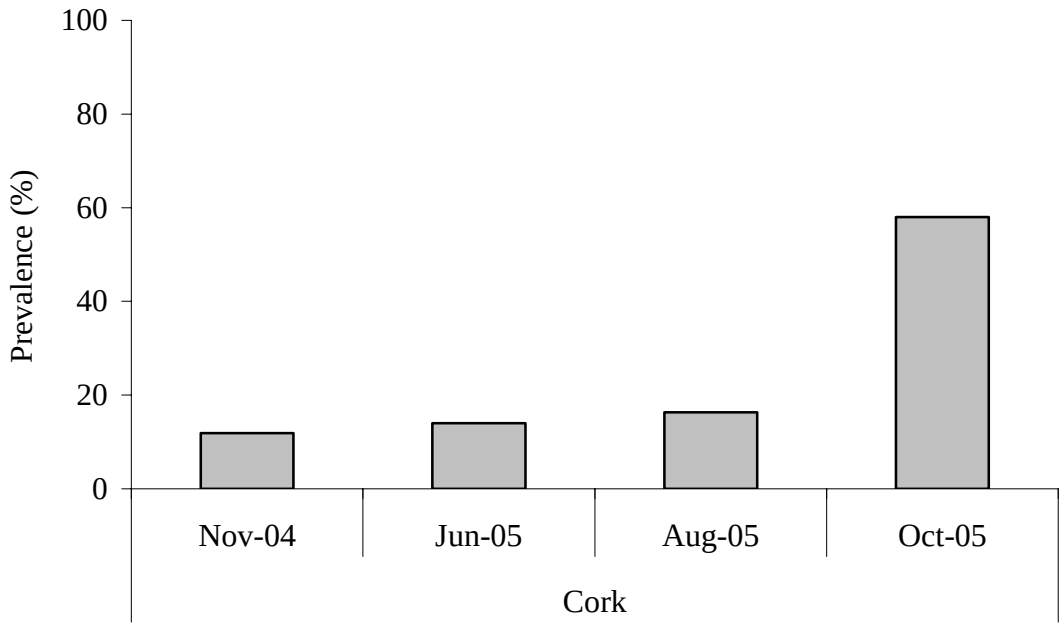
Figure 13. Prevalence of *Haematodinium* in sub-samples from Wexford in 2004 and 2005.



6.6.2 Cork

Figure 14 shows the prevalence of *Haematodinium* in sub-samples was similar in 2004 (12%), June 2005 (14%) and August 2005 (16%) in Cork. Due to cessation of fishing activity for the species, it was not possible to sample during spring 2005. In autumn 2005 this prevalence increased significantly to 58% in sub-samples.

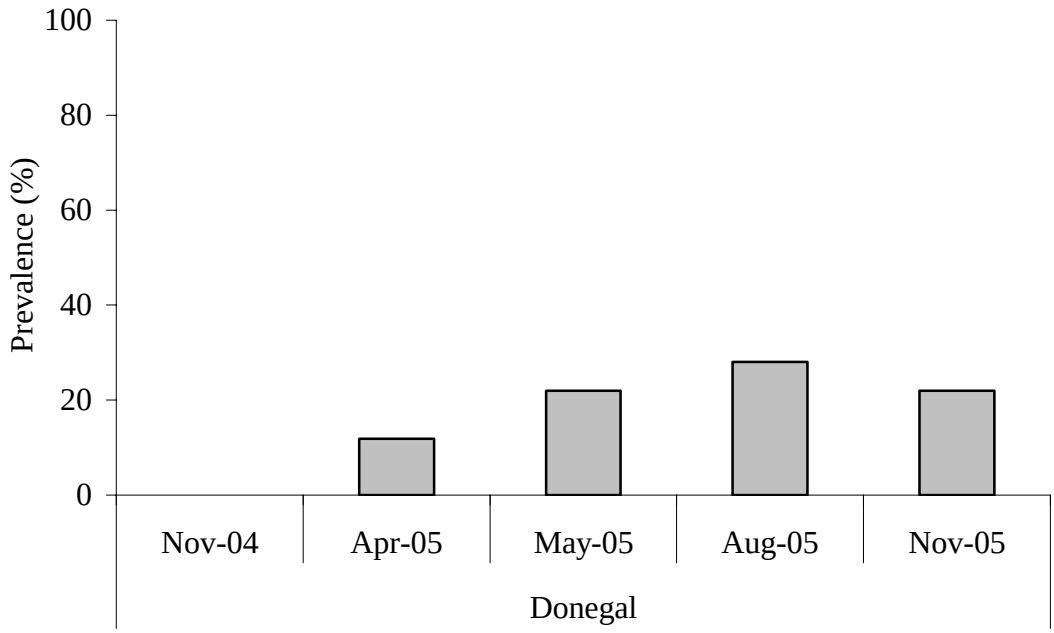
Figure 14. Prevalence of *Haematodinium* in sub-samples from Cork in 2004/2005.



6.6.3 Donegal

No *Haematodinium* infected crabs were detected in November 2004. Infected specimens were detected in sub-samples in April of 2005 (12%) however, Figure 15. This was followed by an increase in the prevalence through May (22%) to August (28%). A prevalence of 22% was recorded in November 2005.

Figure 15. Prevalence of *Haematodinium* in sub-samples from Donegal in 2004/2005.



6.7 Infection Intensity

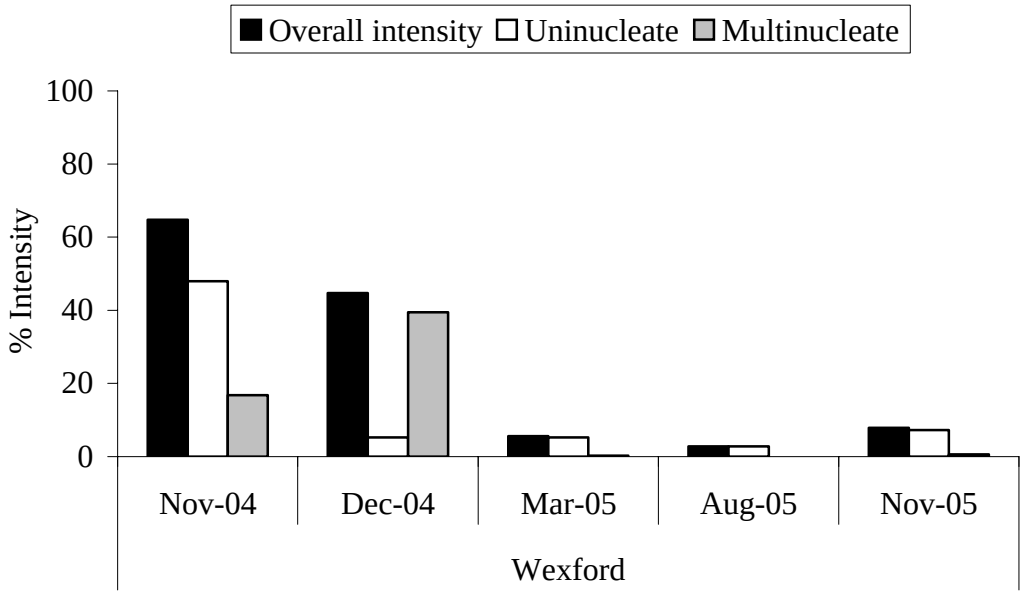
The 'intensity of infection' was calculated by obtaining the proportion of infected cells compared with the total number of cells counted for specimens containing the parasite.

6.7.1 Wexford

Overall intensity was highest in November and December 2004 and subsequently decreased to lowest levels (≤ 5%) in the spring, summer and autumn months of 2005, Figure 16. In November 2004 the parasitic uninucleate cells were dominant, while in December 2004 the majority of parasitic cells were multinucleate. However, it should be noted that in December 2004 sample size of infected individuals was low (n=2)

and may not give an accurate intensity level. Few multinucleate cells were present in 2005 samples.

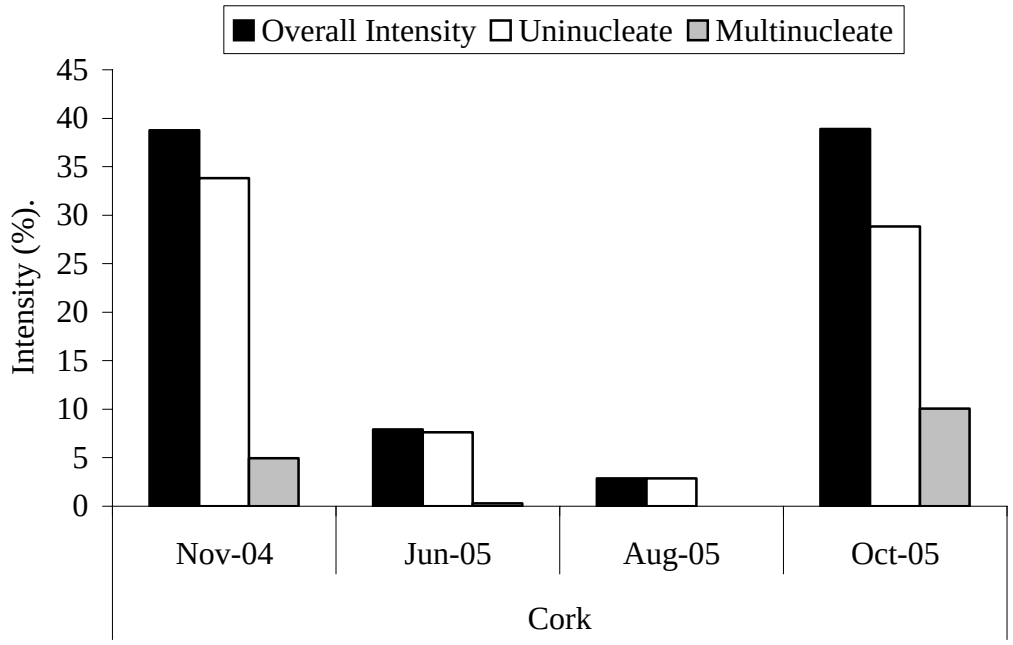
Figure 16. Infection intensity of *Haematodinium* in *Cancer* sub-sampled in Wexford.



6.7.2 Cork

Figure 17 shows that overall intensity was highest in the autumn months (November 2004 and October 2005). Samples in the summer months had low levels of infection. Although uninucleate cells were the dominant *Haematodinium* cell type in the blood during autumn 04 & 05, multinucleate cells were present at low levels. In the summer months no multinucleate cells were recorded.

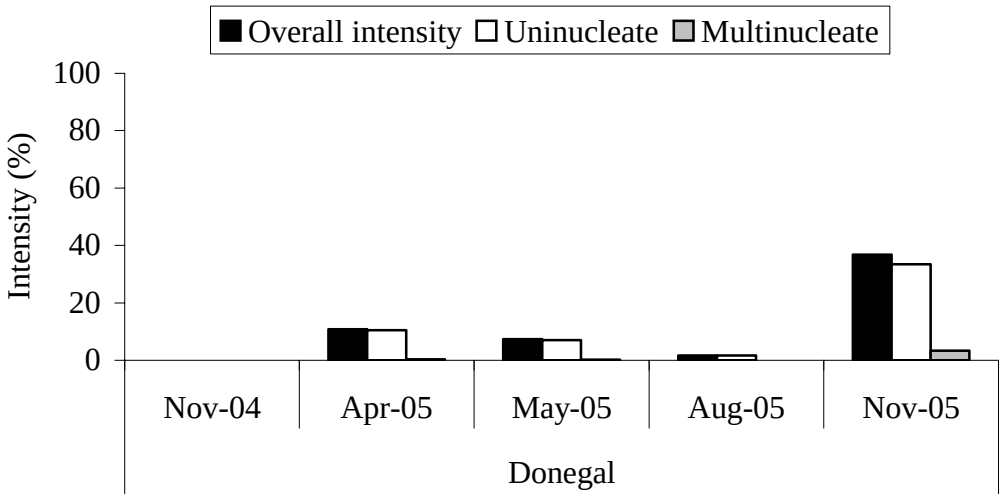
Figure 17. Infection intensity of *Haematodinium* in *Cancer* sub-sampled in Cork. N.B. Note scale change on y-axis when compared to Fig 16.



6.7.3 Donegal

No infection was detected in the autumn of 2004 (Figure 18). Infection levels in the spring and summer of 2005 were low. However, they were within the range noted for spring and summer sampling in Cork and Wexford. In the autumn of 2005 the infection levels increased to the highest recorded (36%). In Donegal, mainly uninucleate cells were observed in infected individuals, however a small number of multinucleate cells were noted in April and November 2005.

Figure 18. Infection intensity of *Haematodinium* in *Cancer* sub-sampled in Donegal.



Until the lifecycle of *Haematodinium* is properly described and understood, particularly in relation to Irish species, information on parasite cell type may be of limited use. There is however some suggestion that high number of uninucleate cells may occur just prior to sporilation, and if verified this may have relevance to future management measures.

6.8 Spatial Variation in Infection Intensity

6.8.1 Local scale

Local-scale spatial analysis of *Haematodinium* infection in brown crab was conducted in the Wexford area in December 2004. Eight areas were selected and a minimum of 15 crabs was sampled from each area. Table 3 shows a summary of the results obtained from the experiment, during which only 3 crab were found to be infected. Variation in the size of crabs between areas was assessed using a one Factor ANOVA

(Table 4). There was no statistically significant difference in the size of crabs (carapace width) sampled.

The variation in the infection intensity between areas could not be assessed in this experiment due to the overall low prevalence of *Haematodinium* in the area at that particular time (Table 2). Three of the eight areas had only one specimen from the 15 with the *Haematodinium* infection, while all other areas had no infected animals present in the sample.

Table 3. *Haematodinium* infection prevalence in an area of the Wexford fishery.

Area	Longitude	Latitude	N	Mean Carapace width (mm)	±SD	Number infected
1	52.1155	6.7683	15	150.6	16	1
2	52.1138	6.6925	15	151	16.7	1
3	52.1083	6.6841	15	150.9	16	0
4	52.1111	6.6922	15	141	11.4	0
5	52.0886	6.6944	15	144.2	14.8	1
6	52.0902	6.6913	15	147.8	17.3	0
7	52.0855	6.6911	15	146.8	17.3	0
8	52.0875	6.6988	15	143.9	15.5	0

Table 4. ANOVA table for comparison of crab sizes from 8 areas in the Wexford fishery.

Analysis of Variance for Carapace width					
Source	DF	SS	MS	F	P
Area	7	1585.6	226.5	0.92	0.494
Error	112	27593.7	246.4		
Total	119	29179.3			

Although a repeat of this experiment was planned for a period when infection intensity was high, this did not occur until the final phase of the project when resources had been directed elsewhere.

6.8.2 Geographic scale

Variation in the infection intensity was compared between the three locations in August 2005 as this was the only occasion when all locations were sampled simultaneously and *Haematodinium* was present. One-way ANOVA (Table 5) showed there was a significant difference in the size (carapace width) of crab sub-sampled between the three locations in this particular month. Further pair-wise comparisons showed that Wexford crabs were significantly smaller than those

sampled in Donegal and Cork, with no significant differences in CW between the later 2 sites. There was no significant difference in the infection intensity between Wexford, Donegal and Cork during this month however (Table 6).

Table 5. ANOVA comparison of the size of crabs sampled in each area during August 2005.

Analysis of Variance for Carapace width					
Source	DF	SS	MS	F	P
Area	2	1768.8	884.4	6.76	0.005
Error	21	2749.3	130.9		
Total	23	4518.0			

Table 6. ANOVA comparison of infection intensity between areas, August 2005.

Analysis of Variance for Infection Intensity					
Source	DF	SS	MS	F	P
Visit	2	18.160	9.080	3.20	0.061
Error	21	59.526	2.835		
Total	23	77.685			

It was difficult to synchronise sampling efforts in each area and control the size distribution of samples available when port sampling and to process large number of samples in the laboratory at any one time. Future efforts to determine geographical variation in infection intensity should aim to achieve a more consistent monitoring programme, but such information is unlikely to feature in management response models. Crab stocks as geographically distinct as those sampled are not/and are unlikely to be managed as one entity in terms of localised/regionalised factors such as disease/parasite outbreak. Geographic variation in intensity is likely to be relevant only in terms of reducing spread when one area remains unaffected.

6.9 Relationship between sex and prevalence

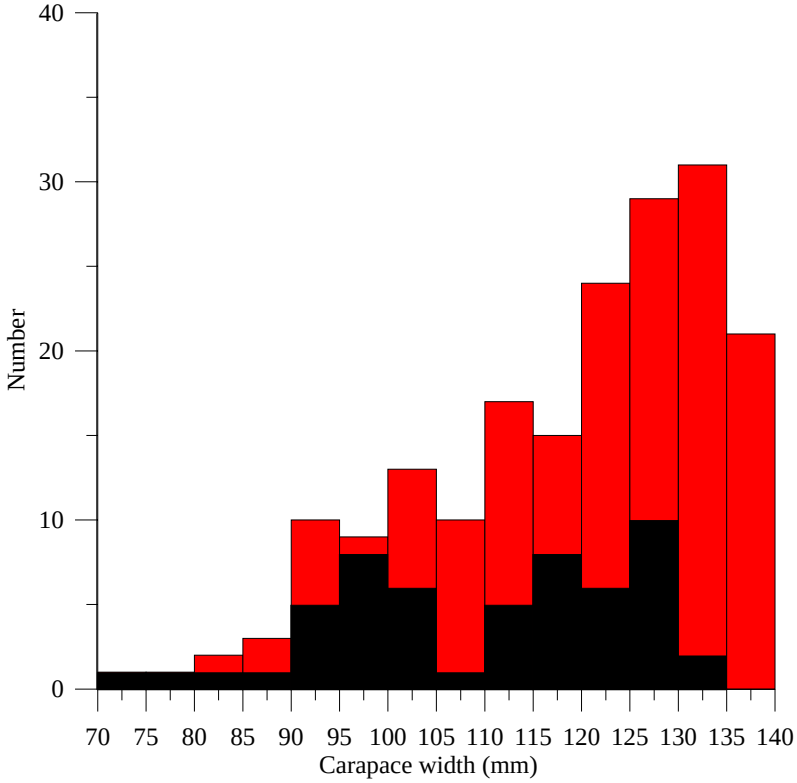
Females dominated the landings of brown crab and this made it difficult to obtain equal numbers of males in this study. As it was deemed desirable to sample individuals of sizes and sex representative of the catch, insufficient numbers of males to conduct analysis on the prevalence and infection intensity where obtained. It is clear however that both males and females are affected by the parasite.

6.10 Macroscopic assessment of infection level on nursery grounds

Of the 186 female intermoult crab from a nursery area in Donegal with the carapace width <140mm that were carefully examined for external symptoms of the disease while at sea during November 2005, 41% were deemed to be infected, Figure 19. A

significantly higher number of individuals were infected in the smallest size classes. Arbitrary division of the crab sampled showed that 64%, 37% and 18% of crab showed clear external symptoms in the <100mmCW, 100-120mmCW and 120-140mmCW size groupings respectively. Microscopic analysis of individuals >140mm carapace width during routine sampling at this time revealed a 22% infection prevalence.

Figure 19. Size-frequency distribution of female *Cancer* <140mm carapace width examined for external symptoms of *Haematodinium* infection at Malin Head, Novemeber 2005. The black bars indicate positive identification of infection.



The results above may indicate that the parasite poses its greatest threat in terms of the potential damage it could cause to recruitment to the fished stock, particularly when 'healthy' animals may have also been infected. Further monitoring of parasite prevalence in nursery areas is warranted, and this should be validated with microscopic analyses.

6.11 Tissue analysis

The extent of tissue degradation was similar in all tissue types. In all but the most severe infections, the organ tissue itself was not badly affected with the majority of the damage being sustained by the surrounding connective tissue. No difference in

parasite cell type was noted between the five stages of infection with both multinucleate and uninucleate stages observed in infected tissues.

With the exception of stage 3, which was not found in claw muscle samples, all stages were found in each of the 5 tissue types. However, only the full 5 stages of the cardiac samples are shown in the following figures to avoid repetition. For the gill, hepatopancreas, claw muscle, and gonad tissue samples, only images of no infection, light infection, and heavy infection are shown. The resolution of the photographic equipment available prevented the preparation of clear images that defined the intermediate stages well.

Healthy cardiac tissue is shown in Figure 20. Stage 1 was defined by the presence of only one or a few parasite cells with no obvious effect on the surrounding tissue (Figure 21). Stage 2 was defined by slightly more parasite cells, Figure 22, covering roughly 20% of the field of view or less and present only within the connective tissue. In stage 3, tissue was moderately degraded and the parasites were very conspicuous in dense aggregations (Figure 23). Complete obliteration of the tissue was associated with stage 4, as can clearly be seen in Figure 24.

Healthy muscle tissue is shown in Figure 25. Infections in the muscle tissue tended to be either very light or very heavy. Large sheets of muscle were left intact in all but the last stages of infection, the parasites occupying the surrounding connective tissue. (Figure 26) The muscle fibres themselves were infiltrated only in the most severe infections, Figure 27.

Healthy hepatopancreatic tissue is shown in Figure 28. Hepatopancreas tissue was severely affected by the parasite, with both cell and tubule structure being degraded. In light infections the parasite cells filled all areas between tubules but did not penetrate them (Figure 29). In the later stages of infection, parasites were present within the lumen of the tubules. Epithelial cells of the tubules had increased vacuolation, Figure 30, as they lost form and disintegrated.

Gonad tissue was the most difficult type of tissue to process histologically. Healthy gonad tissue is shown in Figure 31, and a relatively poor quality image of light infection in Figure 32. Gonad tissue was heavily degraded by severe infection with parasites cells causing morphological disfiguration of the oocytes, Figure 33.

Healthy gill tissue is shown in Figure 34. In light infections, Figure 35, the parasites integrated themselves into the tissue with no noticeable damage to the structure of the organ. In the more heavily infected samples, the filaments were distended by the large numbers of parasite cells, Figure 36, and it seems likely that gas exchange would be hampered at this stage.

Granulomas were present in both gill and hepatopancreatic tissue. Most appeared as swirls of hyalinocytes with melanized centres which appeared to be undergoing necrosis while others were at an earlier stage of development and retained their light blue colouration all the way to the core (hyalinocytes). Figure 37 shows both of these granuloma development stages in the hepatopancreas of a severely infected individual.

Figure 20. Healthy cardiac tissue (x100LM)

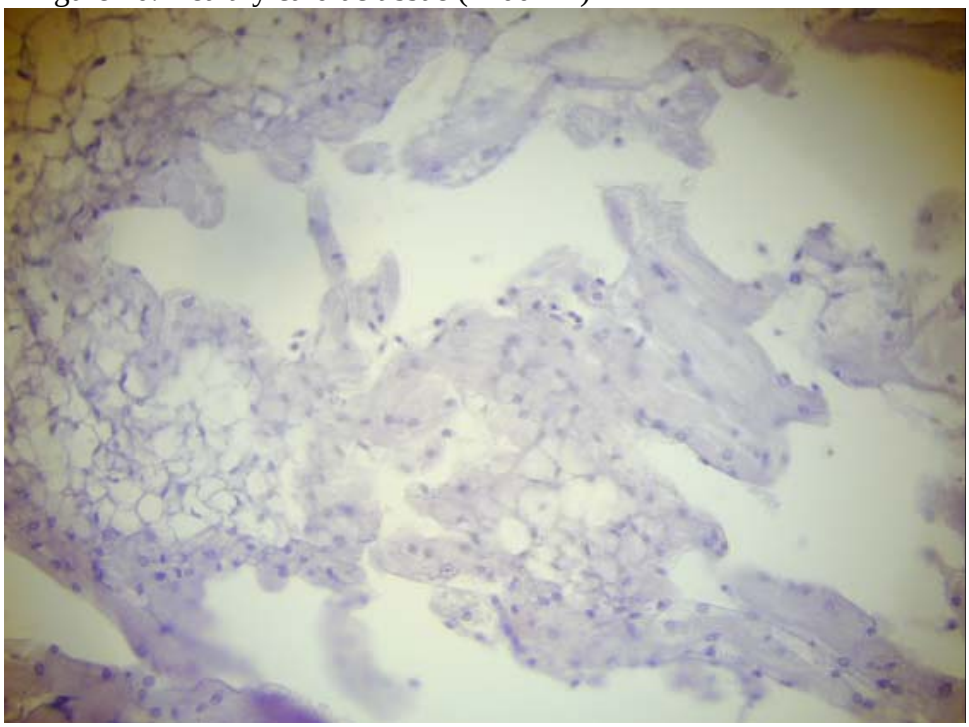


Figure 21. Cardiac tissue at stage 1 with small clusters of 2–4 parasites (arrow) (x100 LM)

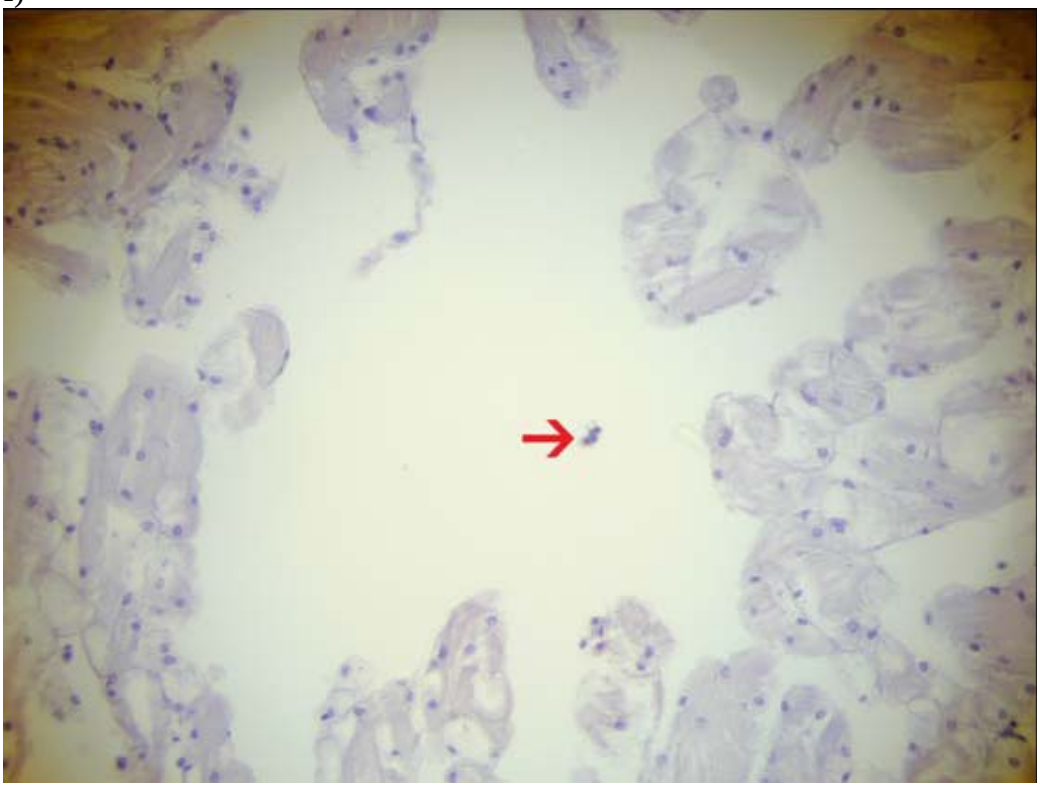


Figure 22. Cardiac at stage 2. Parasites throughout the open spaces of tissue (x100 LM)

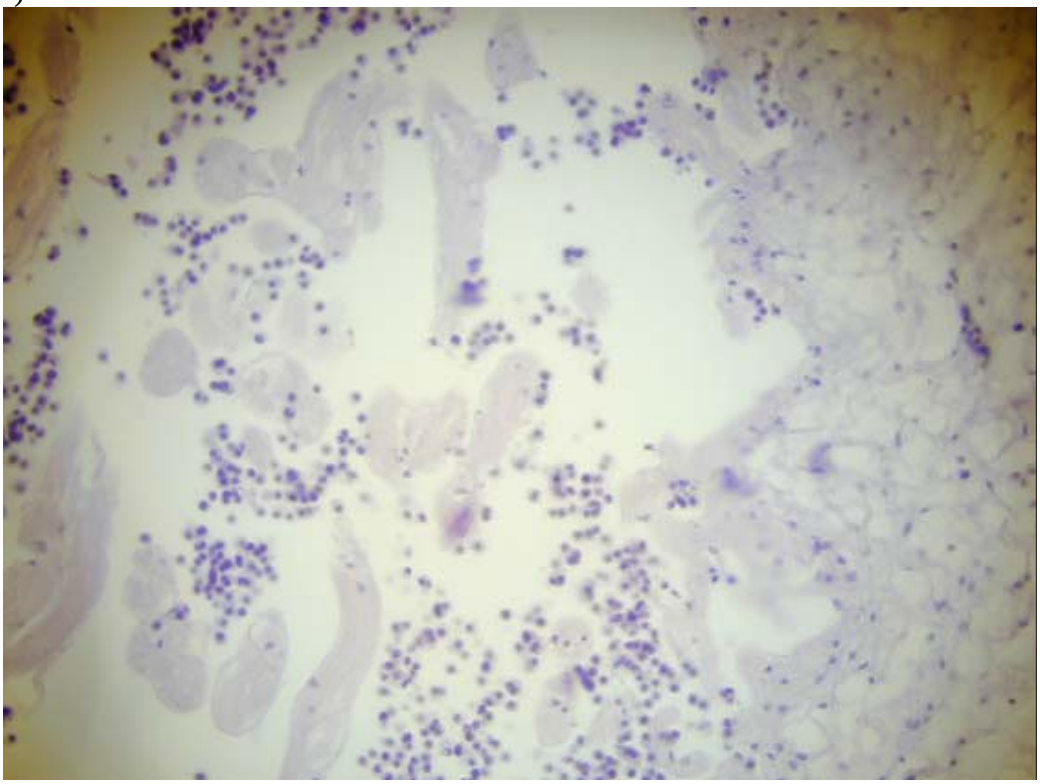


Plate 23. Cardiac tissue at stage 3. Parasites invading some tissue (arrow) (x100 LM).

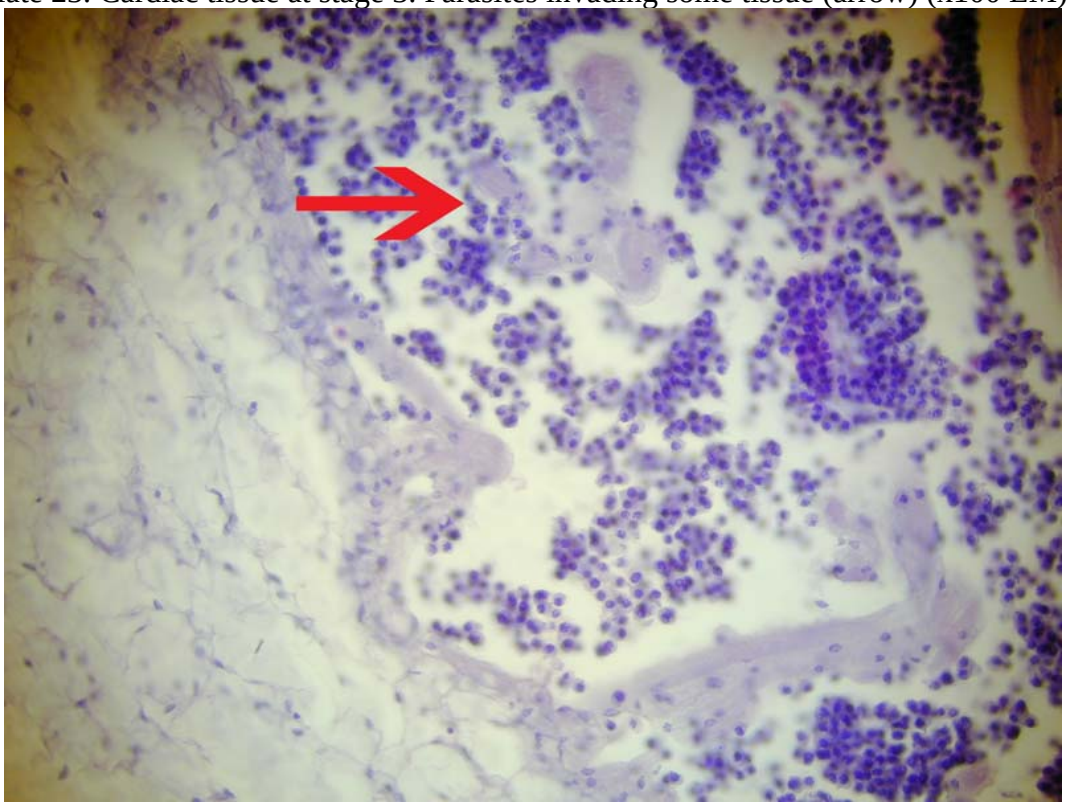


Figure 24. Cardiac tissue at stage 4. (x100 LM)

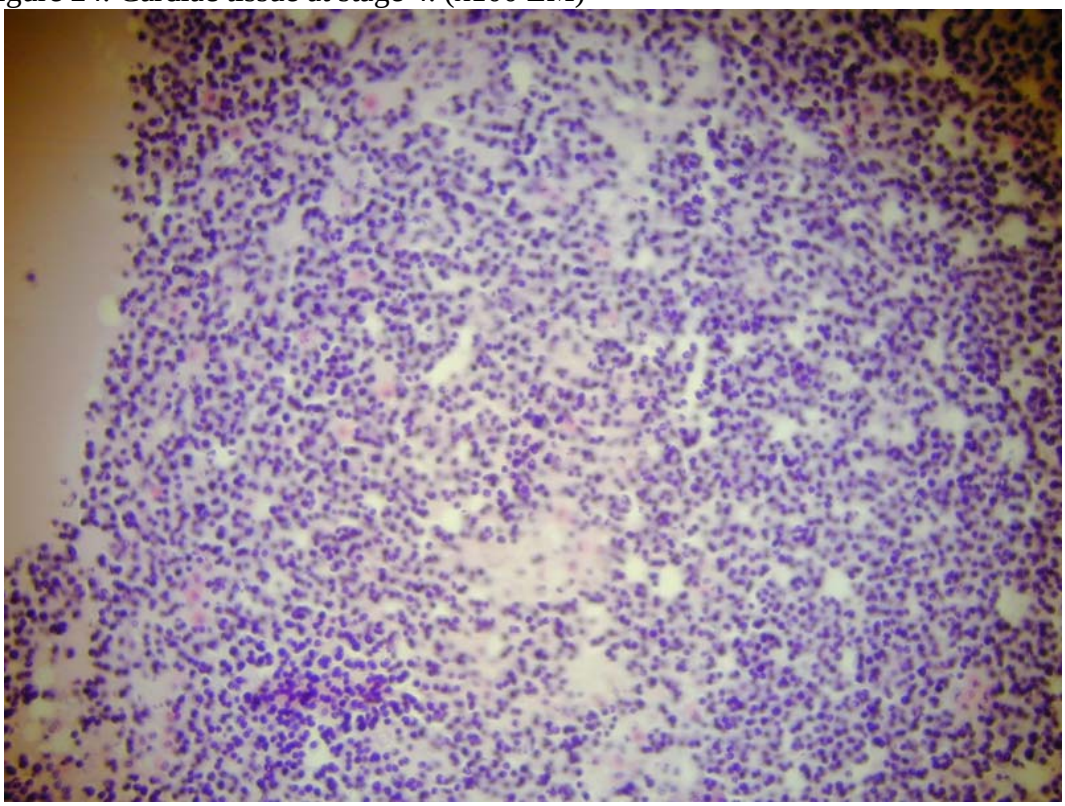


Figure 25. Healthy muscle tissue (x400LM)

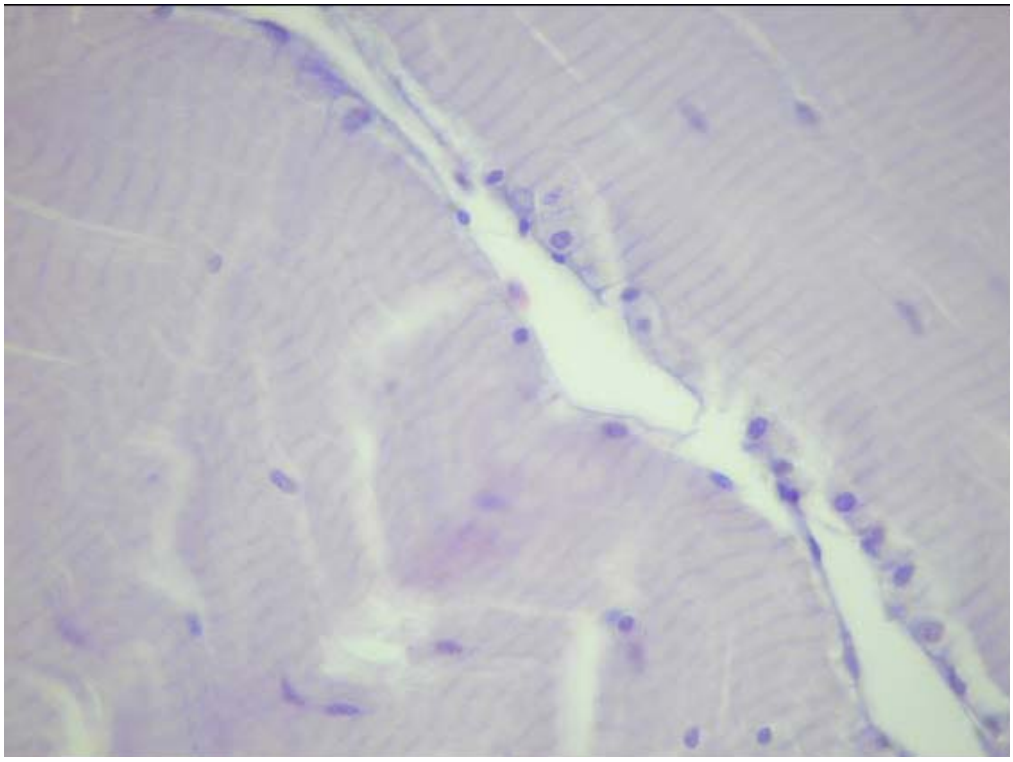


Figure 26. Muscle tissue with light infection, a few parasites (arrows) and intact muscle fibres (MF). (x400LM)

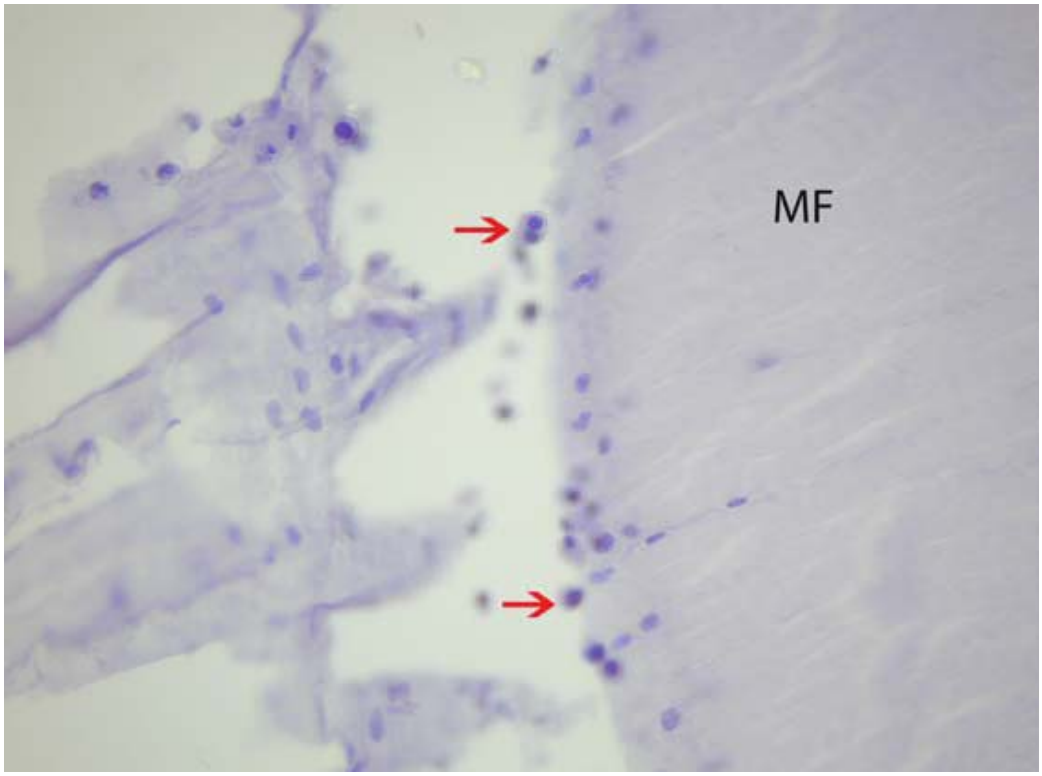


Figure 27. Muscle tissue with heavy infection. Parasites within the tissue (arrow) and degraded muscle tissue (DM). (x400LM)

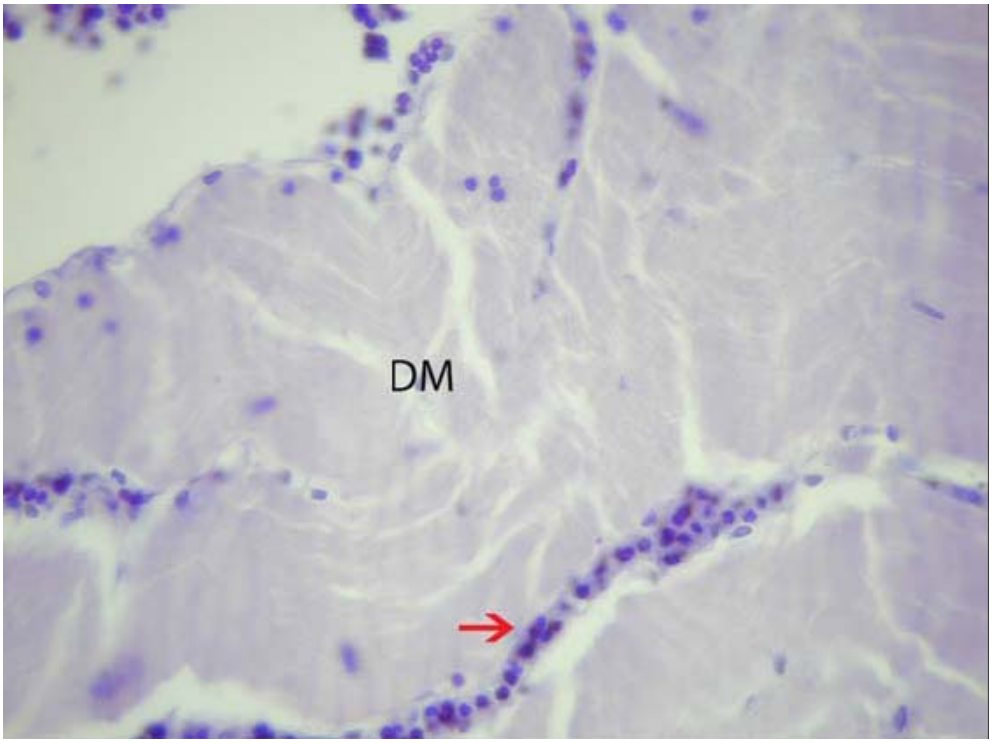


Figure 28. Healthy hepatopancreatic tissue (x200LM)

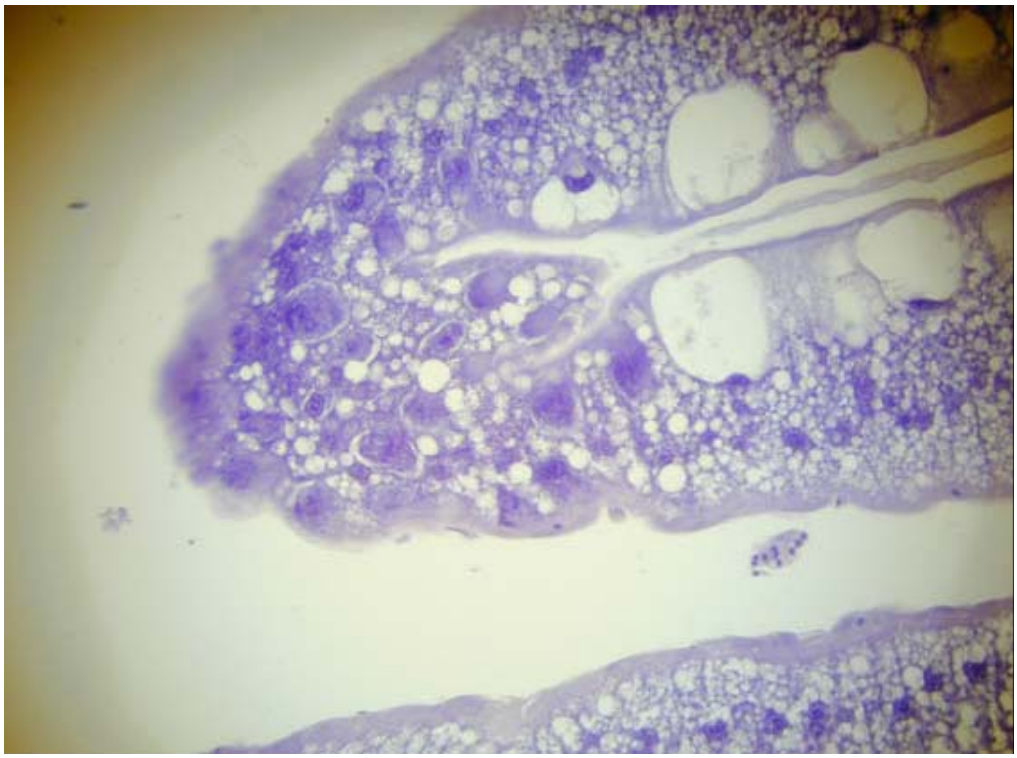


Figure 29. Hepatopancreatic tissue with light infection. Parasites in surrounding connective tissue (arrow). (x200LM)

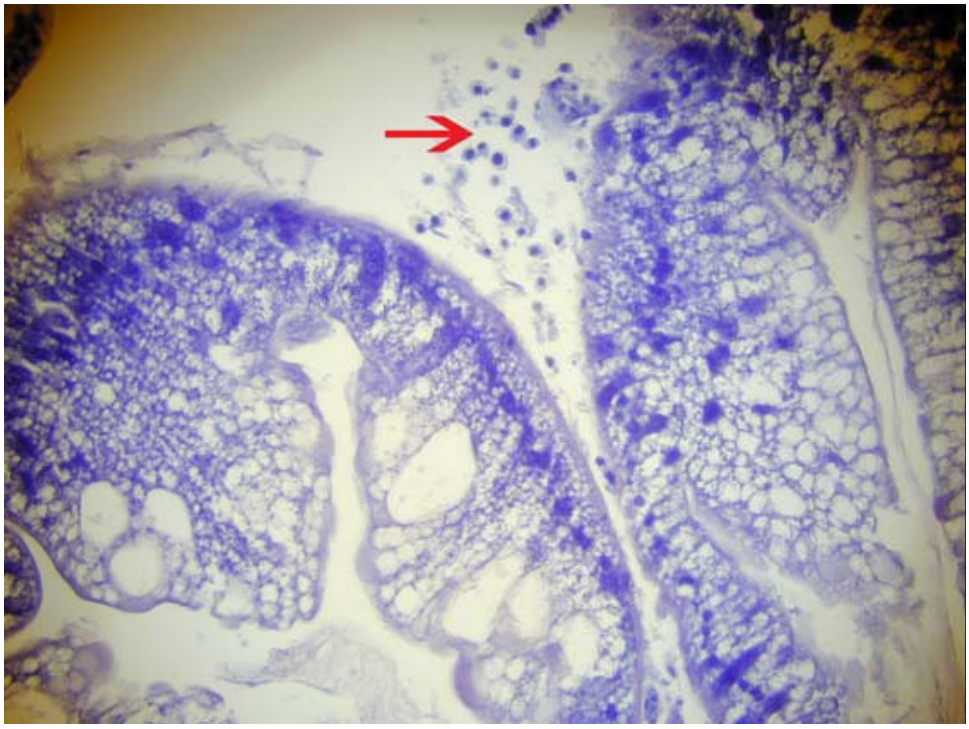


Figure 30. Hepatopancreatic tissue with heavy infection. Parasites completely surrounding the tubules (P) and individuals within the tubule lumen (arrow). Increased vacuolation in epithelial cells (E). (x200LM)

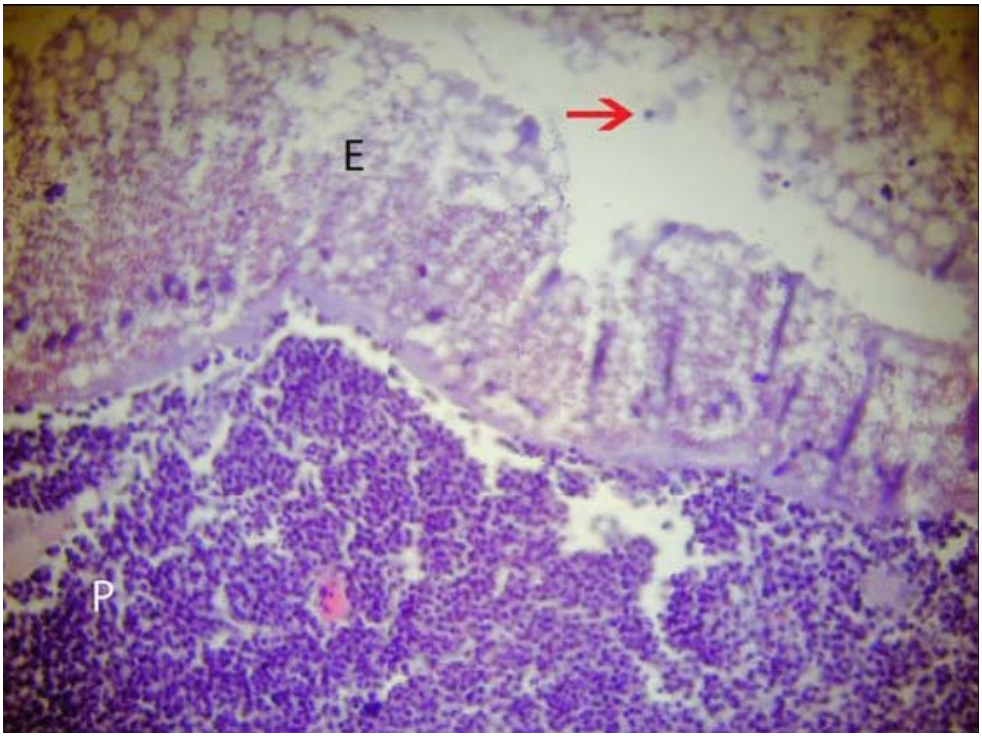


Figure 31. Healthy gonad tissue. Previtellogenic oocyte (red arrow), early stage previtellogenice oocyte (green arrow) (x200LM)

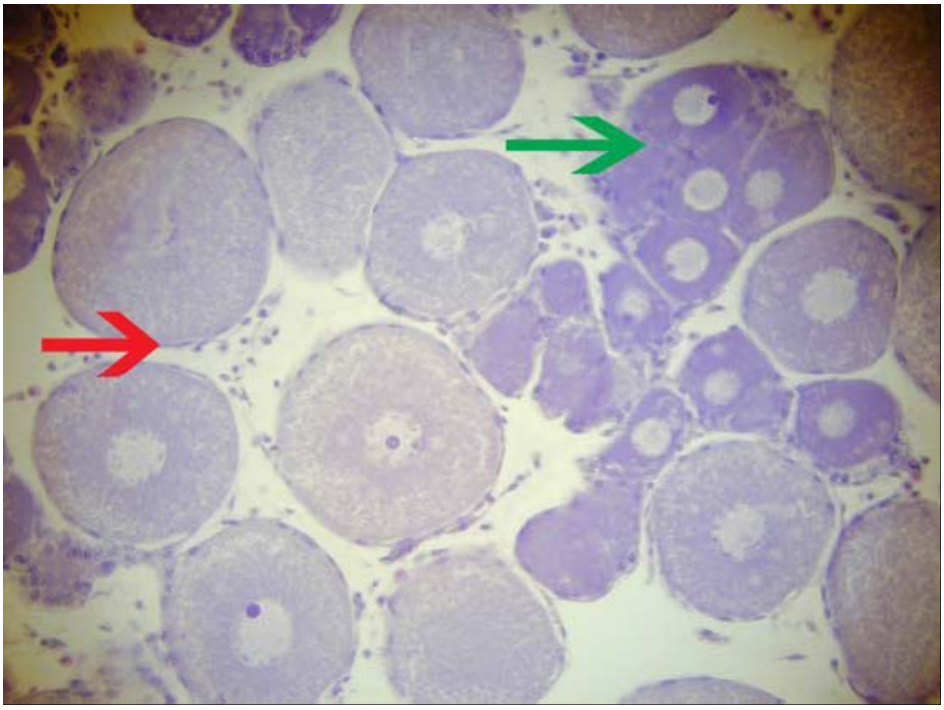


Figure 32. Gonad tissue with light infection. Parasites in spaces between oocytes (x200LM)

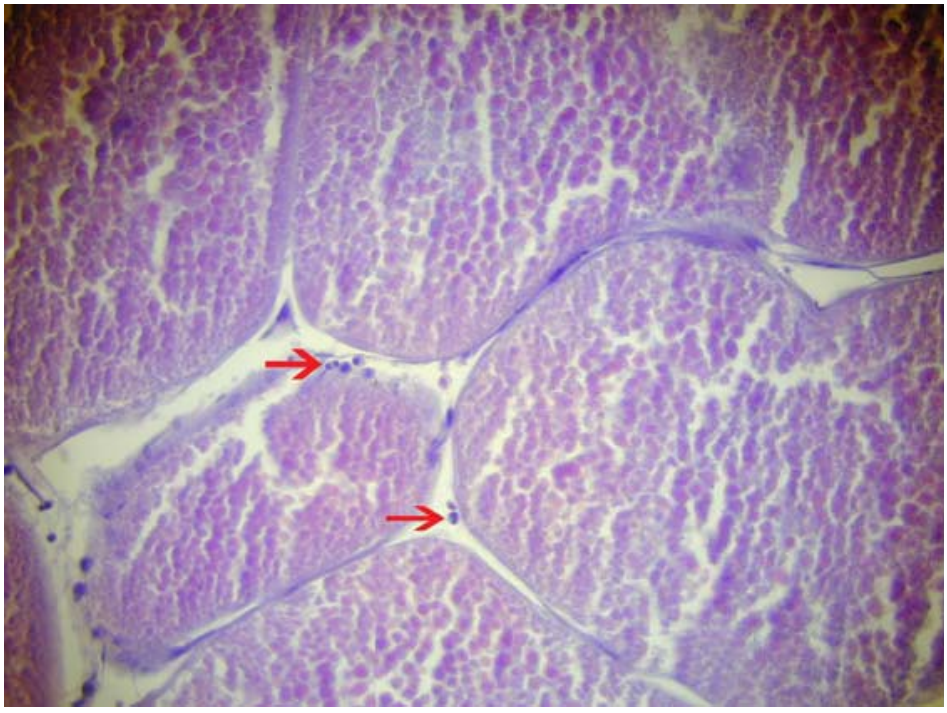


Figure 33. Heavily infected gonad tissue. Parasites filling all areas between oocytes (P). Degenerated early stage previtellogenic oocytes (x200LM)

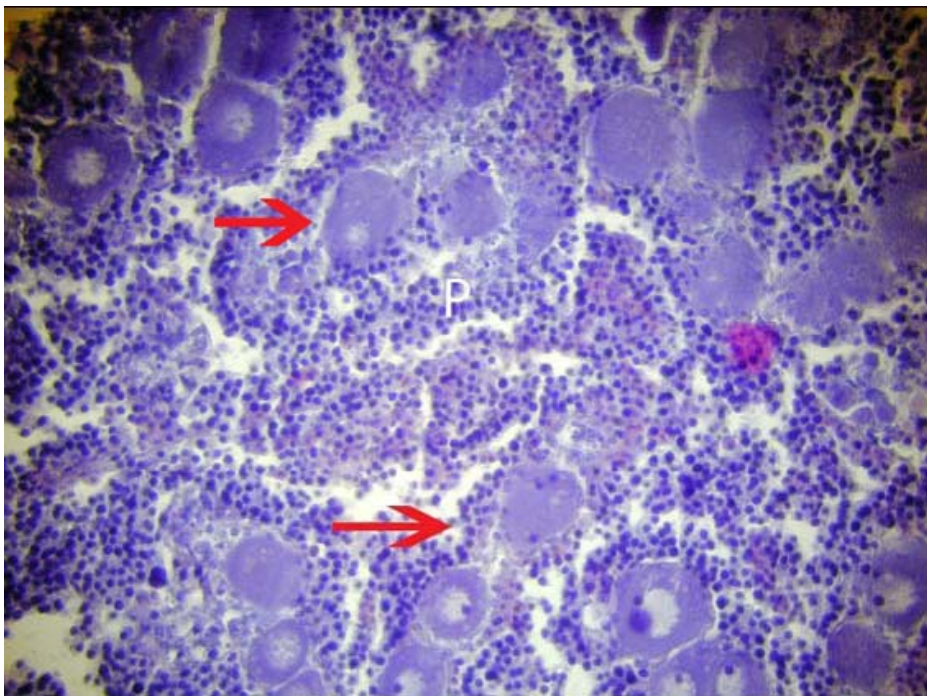


Figure 34. Healthy gill tissue (x200LM)

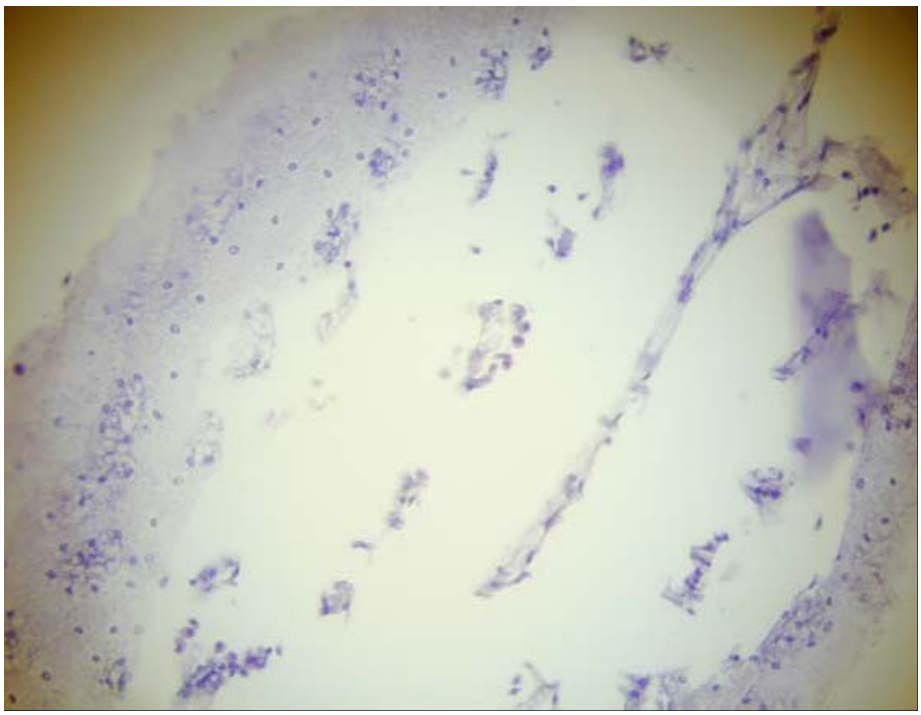


Figure 35. Lightly infected gill tissue. Few parasites found with the filaments (arrows) (x200LM)

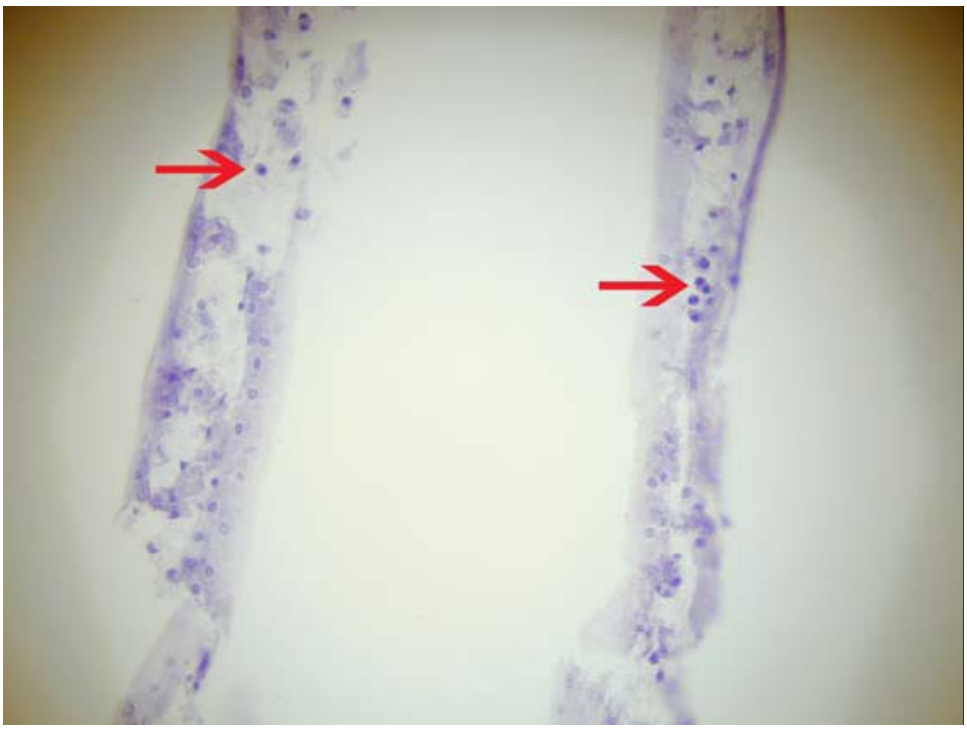


Figure 36. Heavily infected gill tissue. Parasites throughout filament (P) (x200LM)

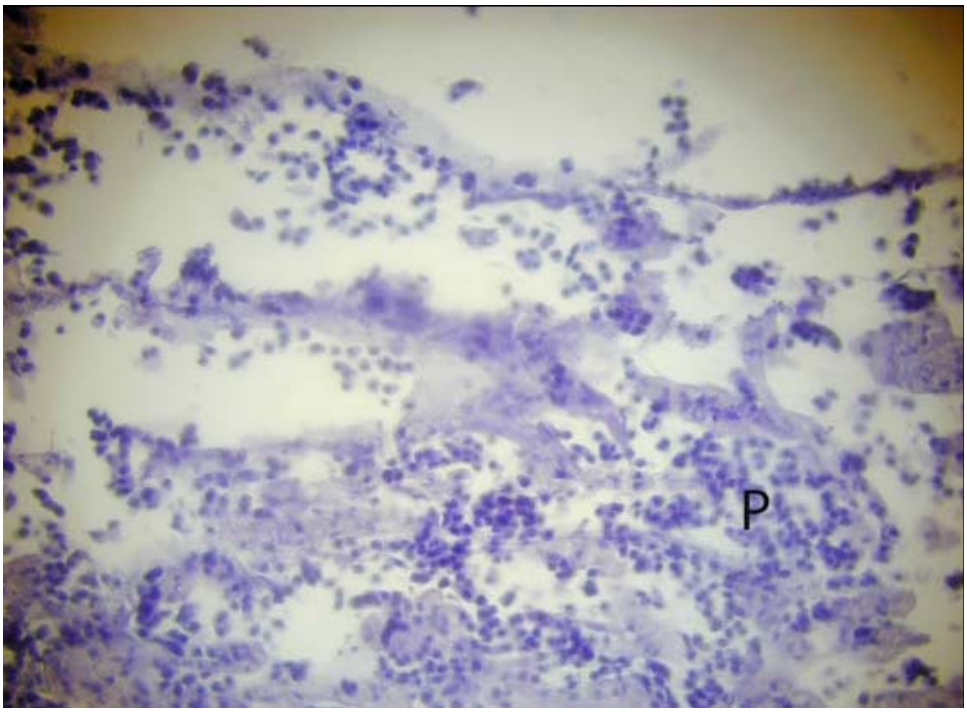
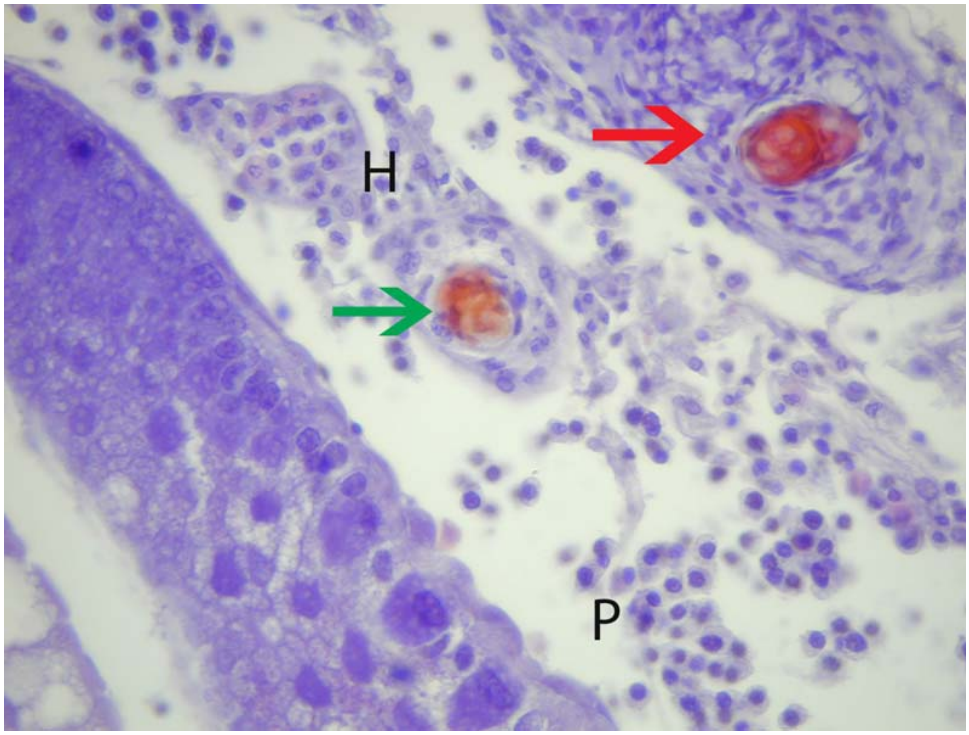


Figure 37. Hepatopancreas with moderate stage 3 infection. Two granulomas, one late stage (red arrow) and one earlier stage (green arrow) granuloma surrounded by haemocytes (H) and parasites (P) (x400LM)



6.12 Cross-infection experiment

After 14days in captivity, few individuals of the original 40 remained. All individuals that had been injected or fed with *Haematodinium* infected muscle had died. Four of the specimens from the former treatment and two from the later showed positive external and internal signs of the type of degeneration associated with infection, but the remainder showed only 'normal' advanced stages of decomposition. Two individuals from the treatment that only exposed the outer surface of the animals to potential infection survived the experiment, as did five specimens from the control. The dead animals from these treatments did not show signs of infection. Microscopic examination of the haemolymph of survivors showed no signs of infection.

Although it appears the first 2 treatments caused potentially fatal infections in at least some individuals, the exact cause of death could not be established. Attempts to extract haemolymph from the decomposing individuals were not made for Heath and Safety reasons. The death of fewer animals in the control treatment could not be attributed solely to lack of parasite action, as they had not been exposed to similar levels of stress as those in treatments. The transport of animals from Donegal to

Wexford without re-tanking facilities to monitor initial condition/survival was considered undesirable, and therefore no firm conclusions were drawn from this pilot experiment. Further experimentation should be conducted with urgency, as the results could have a significant influence of management of *Haematodinium* infections in brown crab (and other crustacean) fisheries.

Although further study is warranted, initial management advice should centre on the restriction of movement of crabs and crab derived products between fishing areas. This should principally focus on the use of dead animals as bait for other species in areas where crab stocks are also of significant value. Although bait is sometimes cooked or frozen prior to arrival at the receiving port, certain species of dinoflagellate are extremely resistant to these treatments when in encysted form.

Future research should examine the potential value of reducing/eliminating clawing in affected areas, closing badly affected areas within fisheries and/or ensuring discards are not delivered to unaffected areas, and the culling and discarding of late-stage infected individuals away from fishing areas or preferably in landfill/incinerators. The potential impact of the parasite on the survival of hosts during medium-term storage and subsequent transport should also be assessed. The histological study conducted suggests that normal physiological functions are probably severely disrupted in the late-stages of infection.

7. References

Briggs, R.P. and McAliskey, M. 2002. The prevalence of *Haematodinium* in *Nephrops norvegicus* from the western Irish Sea. *Journal of the Marine Biological Association of the U.K.* **82:** 427 – 433.

Eaton, W.D., Love, D.C., Botelho, C., Meyers, R.T., Imamura, K. and Koeneman, T. 1991. Preliminary results on the seasonality and life cycle of the parasitic dinoflagellate causing Bitter Crab Disease in Alaskan tanner crabs (*Chionoecetes bairdi*). Journal *of Invertebrate Pathology*. **57:** 426 – 434

Field, R.H., Hills, J.M., Atkinson, R.J.A., Magill, S. and Shanks, A.M. 1998. Distribution and seasonal prevalence of *Haematodinium sp*. Infection of the Norway lobster (*Nephrops norvegicus*) around the west coast of Scotland. *ICES Journal of Marine Science*. **55:** 846 – 858.

Stentiford, G.D., Green, M., Bateman, K., Small, H.J., Neil, D.M. and Feist, S.W. 2002. Infection by a *Haematodinium*-like parasite dinoflagellate causes Pink Crab Disease (PCD) in the edible crab *Cancer pagurus*. *Journal of Invertebrate Pathology*. **79:** 179 – 191.

Stentiford, G.D. and Shields, J.D. 2005. A review of the parasitic dinoflagellates *Haematodinium* species and *Haematodinium*-like infections in marine crustaceans. *Diseases of Aquatic Organisms*. **66:** 47 – 70.

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