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Hematodinium infection seasonality in the Firth of Clyde (Scotland) *Nephrops norvegicus* population: a re-evaluation

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ABSTRACT: *Hematodinium* infections in Norway lobster *Nephrops norvegicus* from the Clyde Sea area (CSA) population, Scotland, UK, have previously been undetected in summer. This study aimed to establish if the CSA is actually devoid of infected *N. norvegicus* in this season. Two PCR assays, an ELISA and 2 tests that detect only patent infection (pleopod and body colour methods) were applied in a 21 mo study. Patent infection was seasonal, appearing predominantly in spring, while subpatent infection diagnosed by ELISA and PCR was highly prevalent in all seasons. Generalised linear modelling supported this assertion, as sampling in September and February significantly increased the probability of finding infected *N. norvegicus* ($p < 0.01$); infections were predominantly subpatent and patent respectively, at these times. Therefore, *Hematodinium* seasonality in *N. norvegicus* populations is likely to have been an artefact of insensitive diagnostic tests. Light *Hematodinium* infections were found using PCR assays when patent infections were at their most prevalent and intense, suggesting that infection develops at different rates in different *N. norvegicus* individuals and that only a portion of the total number of infected *N. norvegicus* die within a single year. These new data were added to a long-term data series for the CSA (1990 to 2008), which showed that after an initial 5 yr epidemic period, prevalence stabilised at 20 to 25%. Comparisons with 'susceptible-infected-recovered/removed' (SIR) models suggest that this high prevalence is maintained through high birth rates of susceptible host *N. norvegicus*.

KEY WORDS: Lobster · Parasite · Diagnostic · Disease · Mortality · Long-term dataset · Dinoflagellate · Monitoring · Fisheries

INTRODUCTION

Commercially valuable Norway lobster *Nephrops norvegicus* populations can be deleteriously affected by the parasitic dinoflagellate *Hematodinium* sp. (Alveolata, Syndinea), which can cause significant mortality and loss of fisheries revenue (Field et al. 1992, Appleton & Vickerman 1998, Briggs & McAliskey 2002). *Hematodinium* sp. (hereafter referred to as *Hematodinium*) has significantly influenced natural mortality in numerous Scottish and Irish *N. norvegicus* populations, in which the parasite occurs annually in up to as much as 40% of trawl-caught *N. norvegicus* (Field et al. 1992, 1998, Stentiford et al. 2001b, Briggs & McAliskey 2002). Diagnosing *Hematodinium* infection in *N. norvegicus* is dependent on the intensity of infection (parasites per infected individual; Bush et al. 1997) *in vivo*. Specifically, *Hematodinium* infections in *N. norvegicus* may be without visual manifestation where *Hematodinium* cells are present in the organs and the haemolymph at low intensities (when <20% of the total cells in the

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haemolymph are typically parasite cells) (defined as subpatent) or they may be visible through aggregations of haemocytes and parasite cells in the pleopod blades and vivid changes in cuticle colour (defined as patent) (Field & Appleton 1995, 1996, Stentiford et al. 2001b,c, Small et al. 2002, Stentiford & Neil 2011). Using patent infections to estimate the prevalence (the proportion infected from a population subsample; Bush et al. 1997) of *N. norvegicus* and indeed, most hosts, harbouring *Hematodinium* spp. infections from a population subsample can have numerous implications. Most importantly, patent signs of infection are known to appear late in the infection cycle (Stentiford & Shields 2005). Therefore these methods may exclude those hosts harbouring subpatent infections. In *N. norvegicus*, this excludes finding infections during the subpatent phase of *Hematodinium* development that precedes patent infection (Field & Appleton 1995, 1996, Appleton & Vickerman 1998, Stentiford et al. 2001c). Therefore, these data may not represent the true infection prevalence at all times.

Using patent signs of infection to diagnose *Hematodinium* infection in *Nephrops norvegicus* show *Hematodinium* infection in *N. norvegicus* to be predominantly a seasonal occurrence in trawl samples, where a peak of infection in late winter and spring is followed by an infection nadir in summer and autumn (Field et al. 1992, Field & Appleton 1995, Stentiford et al. 2001b, Briggs & McAliskey 2002). Such seasonal cycles of infection typify *Hematodinium* infection in most host species (Stentiford & Shields 2005). Furthermore, patently infected *N. norvegicus* spend significantly more time outside their burrows and show reduced tail-flip response compared with uninfected conspecifics (Stentiford et al. 2000a,b, 2001a). Therefore, *N. norvegicus* harbouring patent infections are more available for capture by trawl gear, and any prevalence measures based on trawl samples of patently infected lobsters may be overestimates.

The number of *Nephrops norvegicus* harbouring subpatent *Hematodinium* infections that develop over the summer is unknown. However, PCR and immunoassays indicate that in *N. norvegicus* and numerous other host species, infections exist outside patent infection seasons. For example, using PCR Hamilton et al. (2009) showed that up to 25% of 7 sympatric decapod species (*Cancer pagurus*, *Carcinus maenas*, *Liocarcinus depurator*, *Munida rugosa*, *Necora puber*, *Pagurus bernhardus* and *P. prideaux*) from the Clyde Sea area (CSA), Scotland, UK, can be infected with *Hematodinium* in the summer, whereas peak infection abundance (>25%) was observed in the winter and spring. A Western-blot immunoassay developed by Stentiford et al. (2001c) showed that subpatent infection in *N. norvegicus* from the CSA can be as prevalent in autumn as patent infections are the following spring. However, this study failed to detect infection in summer. The interpretation drawn from the data presented by Stentiford et al. (2001c) was that infection is not carried over in the host *N. norvegicus* from one season to the next (i.e. infection and patency occur in one season), or that a possible alternate host may be involved to complete the life cycle. Interestingly, a small-scale study by Field & Appleton (1996), which used the same antibodies in an immuno-fluorescence antibody test (IFAT), found numerous subpatent infections in summer. However, the IFAT was not employed in a large scale study owing to its time-consuming nature. Small et al. (2002) again used the antibodies to develop a high-throughput ELISA that is 4-fold more sensitive than the Western-blot assay and capable of detecting subpatent infection. Both PCR and ELISA techniques provide scope for elucidating the prevalence of subpatent *Hematodinium* infections in *N. norvegicus*, but neither has yet been used on a large scale.

Our aim in this study was to establish the extent to which the lack of detectable *Hematodinium* infections in *Nephrops norvegicus* outside seasons of patent infection may simply be an artefact of insensitive diagnostic techniques. We hypothesised that high levels of subpatent *Hematodinium* infections are detectable in *N. norvegicus* in all seasons. To achieve this, we studied the CSA *N. norvegicus* population in Scotland where, since its discovery in the late 1980s, annual mortality events associated with *Hematodinium* infection have occurred. A 21 mo prevalence study of *Hematodinium* was undertaken in the present study using the aforementioned ELISA test (Small et al. 2002) and 2 PCR assays (Gruebl et al. 2002, Small et al. 2006). These assays detect subpatent infections. Additionally we used the colour and pleopod methods. Finally, since the study of *Hematodinium* sp. in *N. norvegicus* from the CSA was conducted extensively (yet somewhat intermittently) for over 18 yr, data from the present study were integrated with those collected previously to form a long-term data series.

MATERIALS AND METHODS

Capture of *Nephrops norvegicus*

Trawl hauls were conducted monthly over 2 yr from December 2006 through September 2008 (ex-

cluding August 2008) aboard either the RV 'Aplysia' or the RV 'Aora' from the University Marine Biological Station Millport (UMBSM). A transect to the south of Little Cumbrae Island (55° 46′ N, 4° 59′ W) was used primarily (Fig. 1). However, trawls aboard the RV 'Aplysia' were carried out along different transects in September 2006 (from Great Cumbrae through the Isle of Bute; 55° 46′ N, 4° 59′ W) and in March 2007 (the Largs-Fairlie channel; 55° 46′ N, 4° 52′ W). From December 2006 up to and including August 2007 (hereafter referred to as Year 1) the body colour and ELISA methods were used for diagnosis. Between September 2007 and September 2008, body colour, pleopod, ELISA and PCR methods were used (hereafter referred to as Year 2).

Trawl depths were between 60 and 110 m. Trawls on RV 'Aora' used a dual-purpose commercial net with 80 mm diamond-mesh netting, and trawls on RV 'Aplysia' used a research net with a 50 mm diamond-mesh netting. Trawling duration was approximately 1.5 h in each case. The towing period generally occurred in the morning, between 09:00 and 12:00 h. After the trawl catch was on deck, 100 *Nephrops norvegicus* individuals were immediately taken nonselectively and placed in a tank with flow-through seawater. Each individual lobster was immediately assessed for body colour, sex and carapace length (CL), and a sample of haemolymph (that varied according to *N. norvegicus* size) was then withdrawn from the haemal sinus at the base of the fifth pereiopod. The haemolymph sample was either mixed 1:1 with marine anticoagulant (0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 0.45 M NaCl, 10 mM EDTA, pH 4.6) or allowed to clot, and subsequently either used immediately in ELISA and PCR assays (see below) or frozen at −20°C until use. All haemolymph samples were assayed by ELISA in each month. However, only a proportion of the 100 monthly haemolymph samples were tested by PCR because the quality and quantity of DNA was too low in numerous samples (see below).

Pleopods were viewed with low power microscopy and scored as positive or negative for *Hematodinium* according to the criteria of Field & Appleton (1995). The prevalence of subpatent infections was calculated by subtracting the number of patent infections from the total number of infections.

Haemolymph ELISA

The method used was that of Small et al. (2002) with some modifications. Haemolymph was used at 1:500 with phosphate-buffered saline (PBS) (Sigma-Aldridge) and coated onto microplates at 50 µl per well. The primary anti-*Hematodinium* antibody was diluted 1:5000 in PBS and the secondary antibody (goat against rabbit immunoglobin G [IgG] conjugated with horseradish peroxidase [Sigma-Aldridge]) was diluted 1:10 000 in PBS. A tetramethylbenzidine ultra-slow ELISA substrate for horseradish peroxidase (Sigma-Aldridge) was used to develop the colour reaction, for 5 min at room temperature. Colour intensity was read at a wavelength of 560 nm. Positive controls (haemolymph from *Nephrops norvegicus* with body colour-positive *Hematodinium* infections) and negative controls (uninfected *N. norvegicus* haemolymph) were run in duplicate on each plate.

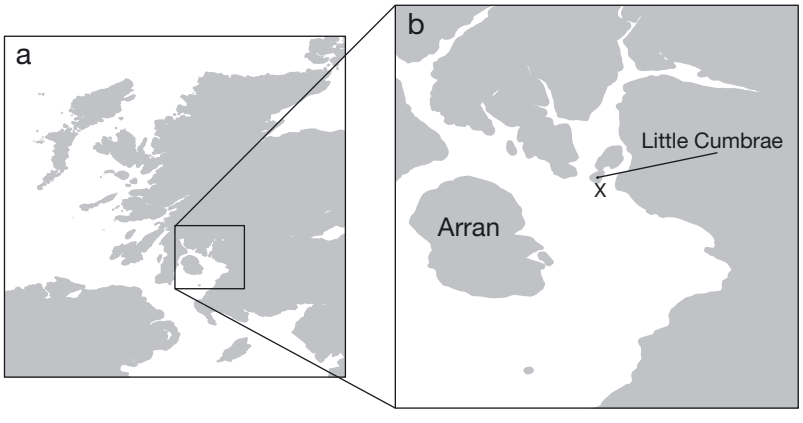

Fig. 1. (a) Western Scotland showing (b) the trawl transect location (X) south of Little Cumbrae Island in the Clyde Sea area used in the present study and also in those studies listed in Table 3

DNA extraction

Haemolymph/anticoagulant samples or frozen clots were initially placed in 250 µl of extraction buffer (50 mM Tris, 5 mM EDTA, 100 mM NaCL, pH 8), 100 µl of 10% sodium dodecyl sulphate (SDS) solution (10% SDS in extraction buffer), 10 µl of Proteinase K (New England Biolabs) (10 µg ml^{-1}) and shaken overnight at 56°C. DNA was collected using both a DNEasy Blood and Tissue Kit (Qiagen) according to manufacturer's protocols, or by the phenol–chloroform extraction method using standard procedures.

The quality of DNA was observed on 1.5% (w/v) agarose gels according to standard procedures, and the quantity was measured using the Nanodrop™ system (Thermo-Fisher Scientific). Once resuspended, if a haemolymph DNA extract was less than ~10 ng μl^{-1} and was accompanied by a visual inspection of quality that deemed no DNA was present (on the gel), the sample was not tested by PCR. However, these samples were still tested by ELISA and visual methods.

PCR screening

Two PCR primer sets were used to screen haemolymph samples collected from trawl surveys. Both primer sets amplified DNA from the rRNA gene complex. Primer Set 1 (developed by Gruebl et al. 2002) employed the Hemat-F-1487 (5′-cct ggc tcg ata gag ttg-3′) and Hemat-R-1654 (3′-ggc tgc cgt ccg aat tat tca c-5′) primers resulting in a 187 bp amplicon. This assay has a reported sensitivity of 1 parasite cell per 300 000 host haemocytes. PCR was performed using 50 µl total reaction volumes; the GoTaq® (Promega) system was used for the PCR mastermix. The mastermix consisted of 10 µl of 5× buffer mix (New England Biolabs), 1.25 µl of the forward and reverse primers (10 µm each), 0.625 µl of dNTPs (10 mM each nucleotide) (New England Biolabs), 0.25 µl *Taq* polymerase (New England Biolabs), 10 ng of the sample DNA and double-distilled water (ddH_20) to 50 µl. PCR thermal cycling was performed as in Gruebl et al. (2002). Samples were stored at 4°C (less than 24 h) or −20°C (long term) until gel electrophoresis was conducted.

Primer Set 2 (developed by Small et al. 2006) was used to screen for the *Hematodinium* genotype infecting *Nephrops norvegicus* and other UK crustaceans (Small et al. 2007, Hamilton et al. 2009). The reported sensitivity of the assay was approximately 0.6 parasites per reaction volume (1 ng genomic DNA). The primer pair 18S F2 (from the 3′ end of the 18S gene; 5′-cag ttt ctg gaa gtg gca gct g-3′) and ITS R1 (3′-gaa ggg aag ggg aga aga agc-5′) resulted in an approximately 380 bp product. The PCR mastermix consisted of 10 µl of 5× buffer mix (New England Biolabs), 1.25 µl of the forward and reverse primers (10 µm each), 0.625 µl dNTPs (10 mM each nucleotide) (New England Biolabs), 0.25 µl *Taq* polymerase (New England Biolabs), 10 ng of the sample DNA and ddH_20 to 50 µl. PCR thermal cycling was performed as in Small et al. (2006) and samples were stored as above.

A 3 µl sample of each PCR reaction was checked for amplification products by 1.5% (w/v) agarose gel electrophoresis and ethidium bromide staining. Images were captured using a BioRad™ gel documentation system (Bio-Rad). Positive and negative *Hematodinium* controls were run alongside all screening PCR reactions. Infected material for positive controls came from heavily infected *Nephrops norvegicus* haemolymph samples and sterile ddH_2O was used as negative control to identify contamination. Haemolymph samples from September and October 2007 and January to August 2008 were screened by both primer sets, whereas those from November and December 2007 were screened by Primer Set 1 and the positives from these months were then screened by Primer Set 2.

Construction of long-term *Hematodinium* data series

Previous data on *Hematodinium* prevalence available for the South Little Cumbrae (SLC) transect over the period 1990 to 2005 were collated from published papers and unpublished reports, in which information on methods of collection and prevalence were documented. Where *Hematodinium* prevalence was estimated by trawling more than once within a month, maximal prevalence values were used. These were added to the data generated in the current study.

Statistical analysis

ELISA and PCR data (see above) from *Nephrops norvegicus* caught in Year 2 (11 mo, exclusive of August 2008) were used to analyse the effect of size, sex and month of capture on the probability of finding infection using generalised linear modelling. Infection was the binomial response variable with size (continuous), sex (categorical) and month (categorical) as covariates. Initially, interactions between sex and size and between month and size were included in the model along with the covariates listed above. These interactions were included for 2 reasons: (1) previous reports of sex and size of infected *N. norvegicus* in the CSA suggested that small *N. norvegicus*, particularly small females, were more predisposed to infection than larger individuals, and (2) female prevalence was much higher in periods where sex ratios are skewed to males. The nonsignificant predictors of infection were systematically

removed until only significant predictors remained. All statistical analyses were performed using the SPSS v. 15.0 statistics package. A significance level of $p < 0.05$ was used throughout.

RESULTS

Nephrops norvegicus population trends over the sampling period

The sex ratios of *Nephrops norvegicus* caught by the trawls over the sampling period November 2006 to September 2008 showed that males dominated the trawl catches for 9 mo of each of the 2 yr, but during the months of May, June and July sex ratios were generally even, with slight female predominance. This pattern has previously been shown to represent the emergence of females from their burrows during the spring, prior to moulting and mating (see Stentiford et al. 2001b, Milligan et al. 2009). After the periods of maximum female emergence (May, June and July), catches were dominated by male lobsters.

Hematodinium infection

Body colour-positive *Nephrops norvegicus* were present in the trawls between February and June of 2007 (4 mo) and between January and July of 2008 (6 mo) (Fig. 2). Prevalence of body colour-positive *N. norvegicus* peaked in the spring at 18% (March) and 11% (February) in the 2 yr, respectively.

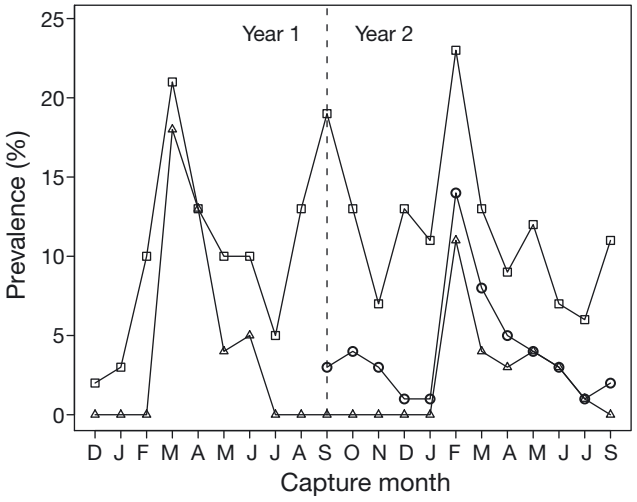

Fig. 2. *Hematodinium* infection prevalence estimates for *Nephrops norvegicus* from monthly trawl subsamples from the Little Cumbrae site between December 2006 and September 2008 (exclusive of August 2008). (□) ELISA, (○) pleopod method and (△) body colour method data are shown. Year 1 and Year 2 are separated by a dotted line, and Year 2 includes September 2007

Pleopod-positive *Nephrops norvegicus* were present in every month in which the pleopod method was used (Fig. 2). Prevalence by the pleopod method showed peak abundance in February 2008 (14%), which closely matched the prevalence of *Hematodinium* by body colour. However, numerous pleopod-positive *N. norvegicus* were present before the body colour method had detected any infection between September and January 2007. In February 2008, when body colour-positive *N. norvegicus* were at maximum prevalence, 3 infected *N. norvegicus* were positive by the pleopod method but not by body colour.

All *Nephrops norvegicus* that were positive by the body colour and pleopod methods were positive by ELISA. Furthermore, the ELISA detected subpatent infection in every month. Infection prevalence by ELISA peaked in late winter and summer of Year 1 and spring in Year 2. The ELISA test also showed reduction in prevalence after the disappearance of *N. norvegicus* that were positive by body colour (in early summer). However, unlike the body colour and pleopod methods, which showed that there was an infection nadir in summer, prevalence by ELISA peaked again in late summer and early autumn.

The emergence of patent infection and the levels of concurrent subpatent infections as diagnosed by ELISA characterised the 2 yr of the study differently (Fig. 2). In the months leading up to maximal body colour-prevalence in Year 1, there were around half the number of subpatent infections seen in the same period in Year 2 (n = 15 and 31, respectively). In the subsequent months, maximal body colour prevalence was seen in March (18%) when 3 *Nephrops norvegicus* that were ELISA-positive had yet to develop the body colour associated with advanced infection. This is considerably lower than the following year when there were still 3 pleopod-positive and 11 ELISA-positive *N. norvegicus* that had yet to develop change in body colour. Also, in Year 2, *N. norvegicus* exhibiting body colour change persisted for longer although the maximal value for body colour prevalence was lower than in Year 1.

Between 54 and 90% of *Nephrops norvegicus* haemolymph samples were examined with PCR assays each month. However, PCR detected infections than were not detected by the ELISA (i.e. PCR positive but ELISA negative), which, when incorporated, increased prevalence values in all but 2 mo (Fig. 3). The ELISA misdiagnosed up to 40% of the

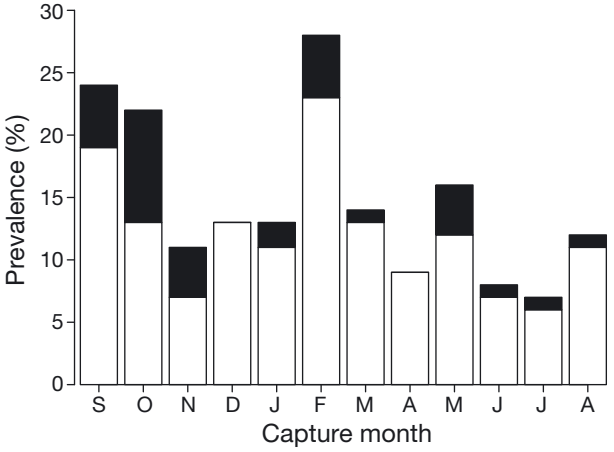

Fig. 3. *Hematodinium* infection prevalence estimates for *Nephrops norvegicus* from the Little Cumbrae site for Year 2 of the study (September 2007 to September 2008, exclusive of August 2008). Prevalence (%) data determined by ELISA (open bars) and PCR (solid bars) are presented. ELISA data includes all *N. norvegicus* that tested positive by the body colour and pleopod methods. PCR positives are in addition to those *N. norvegicus* that tested positive by ELISA

total infected *N. norvegicus* as negative for the parasite. In a single month as much as an additional 9% of trawled *N. norvegicus* were *Hematodinium* infected according to PCR alone (see Table 1, Fig. 3).

Month significantly affected the probability of finding infected *Nephrops norvegicus* (Table 1). Specifically, September 2007 and February 2008 showed significantly more probability of finding infection compared with the other months of Year 2 (Table 2). However, the peak of infection in September 2007 was due to subpatent infections whilst in February 2008 there were twice as many patent infections (Fig. 4). Subpatent infections were present in all seasons (Fig. 4). The level of subpatent infection detected was higher in Year 2 because of the more sensitive PCR assays used.

Sex had no significant effect on the probability of finding *Hematodinium* infection in *Nephrops norvegicus* ($p > 0.05$). However, decreasing body size significantly affected the probability of infection (Fig. 5, Table 1).

Table 1. Relationships between the different diagnostic assays and visual tests for *Hematodinium* in *Nephrops norvegicus*. The columns indicate sampling months (Month) from December 2006 through September 2008 (exclusive of August 2008); the prevalence (%) of *N. norvegicus* with *Hematodinium* infections in trawl subsamples as diagnosed by (A) ELISA (ELISA prev), (C) pleopod (Pleopod prev) and (D) body colour (Body colour prev) methods; (B) the number of *N. norvegicus* that had subpatent infections detectable only by PCR (No. PCR+ & ELISA–). The prevalence of subpatent infections (Subpatent prev) and the total prevalence of infected *N. norvegicus* were derived as indicated by the letters heading those columns

Month	A ELISA prev (%)	B No. PCR+ & ELISA–	C Pleopod prev (%)	D Body colour prev (%)	A+B–C Subpatent prev (%)	A+B Total prev (%)
Dec 06	2	–	–	0	2	2
Jan 07	3	–	–	0	3	3
Feb 07	10	–	–	0	10	10
Mar 07	21	–	–	18	3	21
Apr 07	13	–	–	13	0	13
May 07	10	–	–	4	6	10
Jun 07	10	–	–	5	5	10
Jul 07	5	–	–	0	5	5
Aug 07	13	–	–	0	13	13
Sep 07	19	5	3	0	21	24
Oct 07	13	9	4	0	18	22
Nov 07	7	4	3	0	8	11
Dec 07	13	0	1	0	12	13
Jan 08	11	2	1	0	12	13
Feb 08	23	5	14	11	14	28
Mar 08	13	1	8	4	6	14
Apr 08	9	0	5	3	4	9
May 08	12	4	4	4	12	16
Jun 08	7	1	3	3	5	8
Jul 08	6	1	1	1	6	7
Sep 08	11	1	2	0	10	12

Historical data set

The references from which the long-term data series was constructed are listed in Table 3. The data describe an initial epidemic prevalence in 1990 (the highest prevalence), which oscillated on a declining trajectory until 1995, after which levels decreased to relatively low values for 2 yr (1996 to 1998) (Fig. 6a). However, by 1998 a period of stability in *Hematodinium* prevalence was established. This can be seen by observing the apparently stable annual prevalence peaks (Fig. 6b). For the last 10 yr (1998 to 2008), *Hematodinium* prevalence in the CSA has remained stable, showing consistent annual prevalence peaks of 20 to 25%.

DISCUSSION

In the present study, *Hematodinium* infections in *Nephrops norvegicus* from the CSA were found not to be seasonal occurrences. This classification can only be ascribed to the appearance of patent infections. As

Table 2. Estimates for the logistic regression testing the effects of size and month on the probability of *Hematodinium* infection in *Nephrops norvegicus*. September 2008 is not shown, as this month was the baseline. Sampling in September 2007 and February 2008 and sampling smaller *N. norvegicus* significantly affected the probability of being infected

Variable	Estimate	SE	Wald statistic	df	p	Odds ratio
Month			35.96	11	<0.01	
Sep 07	0.93	0.39	5.50	1	<0.05	2.53
Oct 07	0.63	0.40	2.42	1	>0.05	1.88
Nov 07	0.11	0.46	0.05	1	>0.05	1.11
Dec 07	0.22	0.44	0.25	1	>0.05	1.24
Jan 08	0.40	0.44	0.85	1	>0.05	1.50
Feb 08	1.50	0.40	14.20	1	<0.01	4.52
Mar 08	0.60	0.43	1.91	1	>0.05	1.83
Apr 08	−0.09	0.49	0.03	1	>0.05	0.91
May 08	0.52	0.42	1.54	1	>0.05	1.69
Jun 08	0.11	0.46	0.06	1	>0.05	1.12
Jul 08	−0.34	0.50	0.45	1	>0.05	0.70
Size	−0.08	0.01	21.02	1	<0.01	0.91

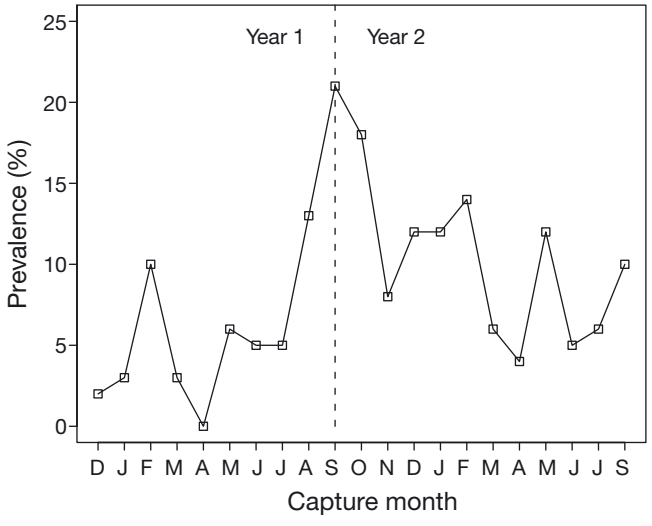

Fig. 4. Prevalence of subpatent *Hematodinium* infections in *Nephrops norvegicus* from the Little Cumbrae site between December 2006 and September 2008 (exclusive of August 2008). Year 1 and Year 2 are separated by a dotted line, and Year 2 includes September 2007

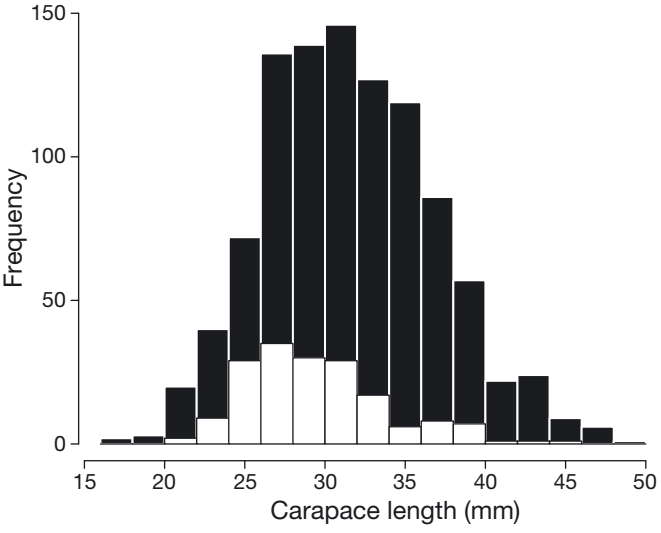

Fig. 5. Carapace length-frequency distribution of uninfected *Nephrops norvegicus* (solid bars) and *Hematodinium*-infected *N. norvegicus* (open bars) for the South Little Cumbrae site over Year 2 of the study (ELISA and PCR data combined)

expected, the body colour and pleopod methods showed infection to be common in winter and spring, which corroborates previous data sets collected using these methods in *N. norvegicus* (Field et al. 1998, Stentiford et al. 2001b, Briggs & McAliskey 2002). However, these methods masked a fluctuating and often high presence of subpatent ELISA- and PCR-positive *N. norvegicus* in all seasons. Our hypothesis that a high prevalence of subpatent *Hematodinium* infection is detectable in *N. norvegicus* in all seasons is supported by these data. Although subpatent prevalence could be as low as 5% it could also be as high as 21% over the period separating patent infection seasons. Such a high prevalence of subpatent infections has not been observed previously during this period in *N. norvegicus* from the CSA. Previously, *Hematodinium* infection in summer was a prominent feature in both years with September 2007 showing a statistically significant peak. In addition, a small-scale tank study undertaken in conjunction with the present study showed that *N. norvegicus* with subpatent infections (as screened by ELISA) in July subsequently developed parasite aggregations in the pleopods, moulted in January the following year, developed (immediately) the body colour change and died in the following February (Beevers 2010). This agrees with previous estimates of *Hematodinium* pathogenesis in *N. norvegicus* and also in the cold-water tanner crab *Chionoecetes bairdi* and snow crab *C. opilio* hosts (Meyers et al. 1990, Appleton 1996, Shields et al. 2005). More experiments are required to substantiate this point, however, as the number of *N. norvegicus* that survived in our small scale study was low. We cannot exclude the possibility that infection and death may still occur in the same year.

For *Nephrops norvegicus*, the ELISA and PCR assays herein have extended the period over which subpatent *Hematodinium* infections are detectable.

Table 3. Source material for the long-term *Hematodinium* data series from the Clyde Sea Area, expressed as number of *Nephrops norvegicus* sampled per month. These are expressed either as the actual number of *N. norvegicus* sampled (denoted by n) or as calculated mean values ($\bar{x} \pm$ SD) of the number sampled monthly

Year	Reference	Diagnostic test	Net mesh size (mm)	No. sampled per month
1990–1991	Field et al. (1992, unpubl.)	Body colour, pleopod	22	$\bar{x}$ = 44.28 (±38)
1992–1998	Field et al. (1998)	Pleopod	22	$\bar{x}$ = 147.15 (±81.66)
1998–2000	Stentiford et al. (2001b)	Pleopod	70	$\bar{x}$ = 221.28 (±62.60)
2000	Small et al. (2002)	ELISA	70	n = 30
2002–2003	Small (2004)	ELISA	70	n = 100
2004–2005	Authors' unpubl. data	ELISA	80	n = 100
2007–2009	Beevers (2010)	ELISA, PCR	80	n = 100

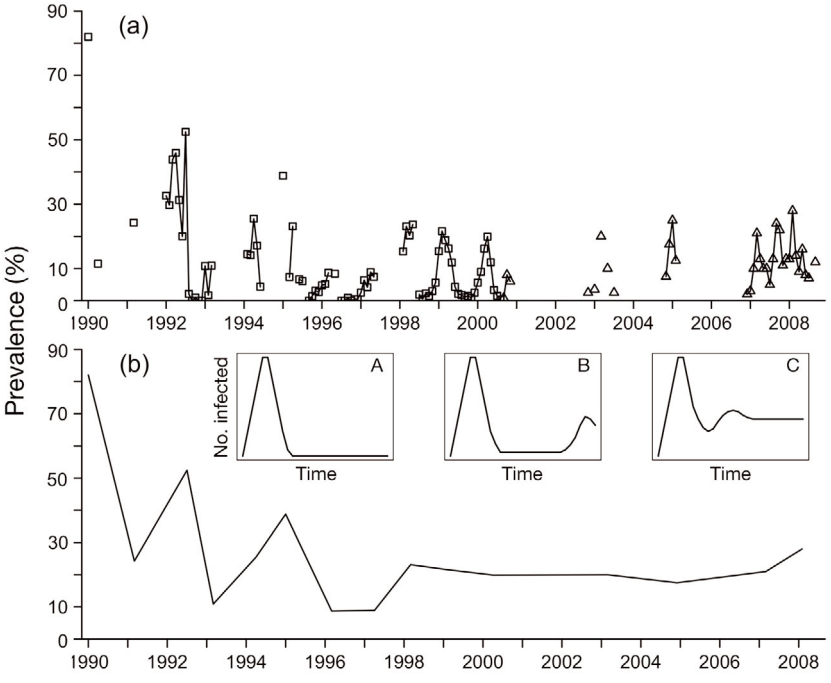

Fig. 6. (a) Plot of the long-term data set collected by the University of Glasgow showing *Hematodinium* prevalence in *Nephrops norvegicus* on the South Little Cumbrae transect of the Clyde Sea area from 1990 to 2008 according to: patent infection methods (□: pleopods, body colour) and methods capable of detecting subpatent infection (△: ELISA, PCR). Monthly samples that are consecutive month by month are linked by lines. Points where no data exist are blank without symbols. (b) Plot of the highest *Hematodinium* prevalence values from each year of the long-term data set. Inset schematics are adapted from Swinton et al. (2002) and are theoretical examples presented for comparison: Schematic (A) describes an epidemic in which host birth rate cannot replace disease-related mortality and the host population and pathogen dies out; Schematic (B) describes an epidemic followed by chance recruitment of susceptible hosts; Schematic (C) describes how a pathogen is maintained endemically because host birth rate is high

Using a Western-blot immunoassay Stentiford et al. (2001c) found that subpatently infected *N. norvegicus* are either absent or undetectable in the CSA in summer. Our data support the latter. The Western-blot immunoassay used by Stentiford et al. (2001c) is 4-fold less sensitive than the ELISA test used in the present study (Small et al. 2002). Therefore, we could detect less advanced infections. In addition, the PCR assays found even more infections in all seasons that were undetected by ELISA. Using PCR tests comparable in sensitivity to those used in the present study, Hamilton et al. (2010) detected summer *Hematodinium* prevalences as high as 25% in numerous sympatric decapod species from the CSA (exclusive of *N. norvegicus*). In that study, peaks of prevalence were in winter and spring. In the present study and also in Hamilton et al. (2010), infection intensity was not recorded in individual hosts. However, we did use 4 diagnostic tests with a wide range of sensitivities on each *N. norvegicus* individual. This allowed us to show that at certain time points, infection intensity may vary considerably and that this may have implications for quantifying levels of *Hematodinium*-associated mortality in this species.

Only a proportion of the total number of *Nephrops norvegicus* hosts that are infected during the patent infection season may succumb to the parasite. In infected hosts, mortality associated with *Hematodinium* is concurrent with the highest infection intensities (Stentiford & Shields 2005). This relationship can predict when mortality will occur (Messick & Shields 2000, Ni Chualain & Robinson 2011). The period in which mortality associated with *Hematodinium* occurs in *N. norvegicus* populations is when body colour-positive lobsters are present in

trawls (Field et al. 1992). This period was from February to July 2008 in Year 2 of the present study. In February 2008 for example, body colour-positive *N. norvegicus* were at their most prevalent (11%) and it was likely that these animals contained *Hematodinium* cells that would soon sporulate and exit the host (Field et al. 1992, Appleton 1996, Beevers 2010). However, there were also 5 *N. norvegicus* that tested positive only by PCR in that month. Such infections could be as low as one parasite cell per 300 000 host haemocytes (Gruebl et al. 2002). If subpatent infections detected by ELISA can take up to 10 mo to reach patency, low level PCR-positive infections may take even longer (Beevers 2010). This challenges previous reports of *Hematodinium* in *N. norvegicus* that suggest all infections culminate in a single bout of high infection in late winter and spring (Field et al. 1998, Stentiford et al. 2001b, Briggs & McAliskey 2002). If it is assumed that infection always leads to host death, then finding PCR-positive *N. norvegicus* in February 2008 suggests that infection develops at distinctly different rates in different *N. norvegicus* individuals throughout the year, and that either only a fraction of the total number of infected *N. norvegicus* found at times of peak prevalence will succumb to infections in that year or that PCR detected very light infections that may be cleared by the *N. norvegicus* host.

Finding previously undetected infections of *Hematodinium* in *Nephrops norvegicus* using ELISA and PCR methodologies may have implications for sex specificity, duration of infection and underlying prevalence. Crustacean parasites with a predilection for certain hosts of a particular sex can exacerbate prevalence, and it was previously reported that small female *N. norvegicus* were more susceptible to *Hematodinium* (Kuris & Lafferty 1992, Field et al. 1998, Stentiford et al. 2001b). However, sex did not affect the probability of *Hematodinium* infection in the present study. We now suggest that males and females are more equal in terms of susceptibility. It is also possible that subpatent infections can persist for long periods in *N. norvegicus* without manifesting as patent infection. Using a nested PCR approach in a Danish *N. norvegicus* population with no history of patent *Hematodinium* infection, Eigemann et al. (2010) found prevalence of subpatent *Hematodinium* infection to be around 65%. Those authors concluded that if the *Hematodinium* DNA amplified by the PCR was from viable cells, infections may not always be fatal. The same may be happening in *N. norvegicus* from the CSA and this may confound attempts to forecast parasite-induced mortality. Finally, patently infected *N. norvegicus* are more available for capture by trawl gear during seasons of patent infection. Therefore, prevalence estimates based on trawl samples of patently infected lobsters may be overestimates (Stentiford et al. 2000a,b, 2001a). However, in summer, when the parasite burden is very low, its effect on host behaviour and physiology will be minimal, thus resulting in no difference in their catchability compared with uninfected lobsters. The prevalence measures made from trawl catches during subpatent seasons may therefore be more accurate representations of underlying prevalence of *Hematodinium* infection.

Prevalence of *Hematodinium* infection in *Nephrops norvegicus* at present is considerably lower than when monitoring in the CSA began. Prevalences reported beginning in 1990 are indicative of an initial 3 yr epizootic (1990 to 1992) in the CSA *N. norvegicus* population. More recently, the observed homogeneity in annual *Hematodinium* infection levels probably represents a (post epizootic) enzootic level. A factor to consider may be that that the 22 mm mesh size used from 1990 and 1998 may have yielded catches with different compositions compared with the 80 mm net used thereafter. However, even by selecting for the *N. norvegicus* most likely to be infected (20 to 30 mm CL, Beevers 2010), it is not currently possible to recreate such prevalence values from a single research trawl. The size and density of *N. norvegicus* on the SLC transect have remained stable over the last 8 yr. Furthermore, the size and prevalence of infected *N. norvegicus* have also remained stable. This is interesting considering the high prevalence of *Hematodinium* in this population and consistently high fishing effort (Stentiford et al. 2001b, Milligan et al. 2009). Comparing these long-term data with 'susceptible-infected-recovered/removed' (SIR) epidemiological models may be useful. SIR models have been used to elucidate similar epidemic–endemic transitions (Swinton et al. 2002) and intriguing similarities exist between the long-term prevalence of *Hematodinium* in the CSA and theoretical populations that support continued high pathogen prevalence through high birth rates of susceptible hosts (see Fig. 6). These data are at the level of detail necessary to observe long-term patterns of *Hematodinium* prevalence in the CSA. Whilst beyond the scope of this work, these data could form the basis of a substantial mathematical modelling exercise that may elucidate how *N. norvegicus* hosts sustain *Hematodinium* at current levels.

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