Assessment of transmissibility of the disseminated neoplasia affecting cockles *Cerastoderma edule* in Galicia (NW Spain)

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Abstract
Disseminated neoplasia (DN) reaches high prevalence in some Galician cockle *Cerastoderma edule* beds, with epidemic dynamics suggesting it is communicable. DN transmission was addressed through various procedures; the disease was transmitted to healthy cockles by co-habitation in tanks with DN-affected cockles and by inoculation of intact haemolymph cells from diseased individuals, but it was not transmitted by inoculation of a filtrate (0.45-µm) of homogenised haemolymph cells from DN-affected cockle individuals. All these results are consistent with recently published molecular evidence of disease transmission between cockles as contagious cancer cells. No evidence of interspecific DN transmission, from cockles to venerid clams, was found either from co-habitation in tanks or analysing clams from beds with high DN prevalence in cockles.

Introduction
Disseminated neoplasia (DN) has been detected in 23 species of marine bivalves with a worldwide geographical distribution (Carballal et al., 2015). This disease has been detected in cockles *Cerastoderma edule* from the coast of France (Poder and Auffret, 1986; Le Grand et al., 2010), Ireland (Twomey and Mulcahy, 1988a; Collins and Mulcahy, 2003) and The Netherlands (Thieltges et al., 2013). It is widely distributed in cockle beds in the Galician coast (Carballal et al., 2001) and its epidemic dynamics suggests it is a communicable disease (Díaz et al., 2016). The cause of DN in bivalve molluscs has been controversial and pollution and infectious aetiology were proposed among others (Barber, 2004; Carballal et al., 2015). The hypothesis of the involvement of an infectious agent was strengthened when the disease was transmitted from affected to healthy individuals in cohabitation experiments in tanks for the cases of *M. edulis* and *M. arenaria* (Brown, 1980; Elston et al., 1988; Mateo et al., 2015) and in the field for *M. arenaria* (Brousseau and Baglivo, 1991, Mateo et al., 2015); however, attempts to transmit DN to healthy cockles by cohabitation with DN-affected cockles failed (Twomey and Mulcahy, 1998a). Transmission was also accomplished by inoculation of haemolymph from diseased individuals into healthy ones in *M. edulis*, *M. arenaria* and *C. edule* (Elston...
et al., 1988; McLaughlin et al., 1992; Sunila, 1992; Twomey and Mulcahy, 1988b; Collins and Mulcahy, 2003; Mateo et al., 2015). The observation of viral particles in cancer cells of *M. arenaria* (Oprandy et al., 1981) led to the hypothesis of a viral aetiology. This hypothesis was reinforced by detecting reverse transcriptase activity in DN-affected *M. arenaria, C. edule* and *Polititapes aureus* (=*Venerupis aurea*) (Medina et al., 1993; Romalde et al., 2007; Manso et al., 2012). Furthermore, viral replication was induced in *M. arenaria* with injections of 5-bromodeoxyuridine (Oprandy and Chang, 1983) and the same inducer was used to activate neoplasia in clams *M. arenaria* from beds with different DN prevalence (Taraska and Böttger, 2013). The hypothesis of viral aetiology was further tested by inoculating a filtrate of homogenised haemolymph cells from diseased individuals into healthy ones; transmission of DN by this “cell-free” procedure was reported in *M. edulis* (Elston et al., 1988), *M. trossulus* (Kent et al., 1991), *C. edule* from Ireland (Collins and Mulcahy, 2003) and *M. arenaria* (Walker et al., 2009; Taraska and Böttger, 2013). Attempts for mollusc interspecific DN transmissibility, from affected cockles to three venerid clam species, was assessed by cohabitation in tanks and by diagnosing clams collected from a shellfish bed with high DN prevalence in cockles. The results of these experiments had remained unpublished but, because of their consistence with recent molecular evidence of DN transmission between cockles as contagious clonal cancer cells, they are now reported.

**Materials and methods**

**Biological materials**

Cockles were collected in April 2004 from two beds in Galicia (NW Spain), one located in Sarrido (ria of Arousa), with high DN prevalence (up to 36%, Díaz et al., 2016), and another bed in Testal (ria of Muros) with low DN prevalence (up to 3%). Additionally, cross-cut carpet shell clams *Ruditapes decussatus* were taken from a shellfish bed located in Aldán Ria (NW Spain), where DN has never been detected. All those cockles and clams were used to perform disease transmission trials.

In 2004, when the aetiology of DN in *Cerastoderma edule* was unknown, the transmissibility of this disease was assessed through attempts to transmit it to healthy cockles by cohabitation with DN-affected cockles in tanks, by inoculation of intact neoplastic cells from diseased cockles, and by inoculation of a filtrate (0.45-µm) of homogenised haemolymph cells from DN-affected individuals. Additionally, interspecific DN transmissibility, from affected cockles to three venerid clam species, was assessed by cohabitation in tanks and by diagnosing clams collected from a shellfish bed with high DN prevalence in cockles. The results of these experiments had remained unpublished but, because of their consistence with recent molecular evidence of DN transmission between cockles as contagious clonal cancer cells, they are now reported.

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**Diagnosis**

Biopsy of every cockle and clam used in the transmission trials to perform non-lethal diagnosis involved taking a haemolymph sample
from the posterior adductor muscle of each cockle, using a syringe with a needle, through a notch made in the shell with a carpenter rasp. The haemolymph was used to produce a cell monolayer onto a slide by cyto-centrifugation (92 x g, 5 min, 4 °C), which was fixed and stained with Hemacolor® (Merck) kit and examined with light microscopy for DN diagnosis. The cockles were ranked according to a scale of disease severity: Non-affected (N0), low severity (N1), moderate severity (N2), and high intensity (N3) (Díaz et al., 2010).

Additionally, cockles and clams found dead in the experimental trials and those that survived at the end of the trials were diagnosed with a standard histological procedure (Carballal et al., 2001). Cockles were classified according to a DN severity scale as follows: light DN, when only isolated neoplastic cells were observed in the connective tissue of some organs; moderate DN, when small foci of neoplastic cells were observed in the connective tissue and haemolymph vessels throughout organs; and heavy DN, when neoplastic cells occurred in massive concentrations through all organs with the destruction of normal tissue architecture. The clams taken from the bed located in Sarrido were also processed by this histological procedure for diagnosis.

**Laboratory transmission trials**

Four DN transmission trials were performed in laboratory conditions, three intraspecific (cockle to cockle) trials and one interspecific (cockle C. edule to clam R. decussatus) trial. Intraspecific trials included co-habitation of healthy and DN-affected cockles, inoculation of haemolymph cells and homogenised haemolymph cells from DN-heavily affected cockles into healthy cockles. All the trials were performed in tanks (25 litres); the tanks used in the co-habitation trials were supplied with seawater pumped from the sea in open circuit. In the inoculation trials, a closed circuit with filtered (0.22 μm) UV-sterilised, aerated seawater was used; cultured algae (mix of Isochrysia aff. T_ISO galbana, Tetraselmis suecica and Skeletonema costatum) were supplied as food. The tanks were checked daily and moribund or dead individuals were taken and processed for diagnosis by histology. The trials ran until all the individuals died except in the intraspecific co-habitation trial that finished after 116 days, when some cockles were still alive.

**Intraspecific transmission trial by co-habitation**

Three tanks were used, one control tank (C1) with 25 non-affected cockles, and two “challenge” tanks (Ch1, Ch2) each enclosing 25 non-affected cockles and 15 DN-affected ones. At day 68 from the beginning of the trial, 3 new DN-affected cockles were added to the tank Ch1 and 11 to the tank Ch2 because many DN-affected cockles set at beginning had died; the reason of adding a different number of DN-affected cockles to each of those tanks was to keep the same ratio DN-affected/ non-affected cockles in those tanks.

**Inoculation of neoplastic cells into healthy cockles**

Four tanks were used, two control (C2, C3), and two “challenge” tanks (Ch3, Ch4). Thirty non-DN-affected cockles were set in each tank. The cockles in the control tanks were injected in the adductor muscle, through a notch in the shell, with 0.1 mL of a suspension of normal cockle haemocytes (3x10⁶ cells/mL) in filtered,
autoclave-sterilised seawater; the cockles in the “challenge” tanks were injected with 0.1 mL of a suspension of haemolymph cells from DN-affected cockles (3x10^6 cells/mL) in filtered autoclave-sterilised sea water. The suspension of cockle haemocytes was produced by collecting and pooling haemolymph from healthy cockles (N0), whereas the suspension of haemolymph cells from DN-affected cockles was produced by collecting and pooling haemolymph from DN-heavily affected cockles (N3); in both cases, the haemolymph was centrifuged (800 x g, 10 min, 4 °C) and the sediment (cell fraction) was used.

**Inoculation of a filtrate of homogenised neoplastic cells**

Four tanks were used, two control (C4, C5) and two “challenge” tanks (Ch5, Ch6). Thirty non-DN-affected cockles were set in each tank. The cockles in the control tank were injected in the adductor muscle with 0.1 mL of a filtrate of homogenised normal haemocytes from healthy cockles; cockles in the “challenge” tanks were injected with 0.1 mL of a filtrate of homogenised haemolymph cells from DN-affected cockles. The filtrates of homogenised normal haemocytes and homogenised haemolymph cells from DN-affected cockles were produced by collecting haemolymph from healthy cockles (N0) and from heavily affected cockles (N3), respectively; the haemolymph samples were centrifuged and the pellets resuspended (3x10^6 cells/mL), as described above; then cells were sonicated (Branson Sonifier 450, equipped with a 3-mm tapered microtip, 15W for 15 sec, with the tubes containing the cells maintained in crushed ice), the resulting suspension was centrifuged (3000 x g, 10 min., 4 °C) and the supernatant was filtered (0.45-µm) under sterile conditions.

**Interspecific transmission trial by co-habitation**

Four tanks were used, two control tanks (C6, C7) with 30 *R. decussatus* each, and two “challenge” tanks (Ch7, Ch8) each having 30 *R. decussatus* plus 25 DN-affected cockles. The clams were kept in tanks after all cockles died to allow disease progression in clams if transmission had occurred.

**Statistical analysis**

Differences in survival between cockle DN severity stages in the intraspecific transmission trial by cohabitation were evaluated with the nonparametric linear Mantel-Cox test (significance level at alpha = 0.05) using SPSS 20 software.

**Results**

No case of DN was detected in cockles of the control tank corresponding to the intraspecific transmission trial by co-habitation, whereas it was detected in 21% and 17% of the cockles initially diagnosed as non-affected in “challenge” tanks Ch1 and Ch2, respectively (Table 1); five of them showed light DN, 3 moderate DN and 1 heavy DN, while 3 cockles could not be processed because their tissues were too degraded. Regarding the evolution of DN severity in cockles that were already affected at the beginning of the trial, 89% of them showed the same severity when examined after death or at the end of the trial; DN severity increased in 8% of the cockles and decreased in one cockle (3%), from moderate to low severity. No case of absolute DN remission was found. This trial ran for 116 days.

No case of DN was detected in cockles of the control tanks of the trial involving inoculation of neoplastic cells, whereas the disease was
detected in 14% and 20% of the cockles in “challenge” tanks Ch3 and Ch4, respectively (Table 1). The first DN-affected cockle was detected 3 days after inoculation, with light DN severity. DN reached light severity in 4 cockles inoculated with neoplastic cells, moderate severity in 1 cockle and high intensity in 3 of them. This trial ran for 32 days and 32 cockles could not be diagnosed when removed from tanks because their tissues were too degraded.

The interspecific transmission trial by co-habitation ran for 273 days and no DN-affected clam was detected (Table 1). There were live cockles in the “challenge” tanks until day 165 from the beginning. Five clams could not be diagnosed because their tissues were too degraded.

Table 1. Results of transmission trials. A: clams *Ruditapes decussatus*; B: non-DN-affected cockles *Cerastoderma edule*; N: DN-affected cockles (donors).

<table>
<thead>
<tr>
<th>Type of trial</th>
<th>Duration (days)</th>
<th>Tank code</th>
<th>Initial no. of individuals</th>
<th>Final diagnosis of initially healthy individuals:</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No. DN-affected indiv. / No. examined indiv.</td>
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<tr>
<td>Co-habitation</td>
<td>116</td>
<td>C1 25B</td>
<td>0/25</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Ch1 25B + 15 N</td>
<td>5/24</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ch2 25B + 15 N</td>
<td>4/23</td>
<td></td>
</tr>
<tr>
<td>Intraspecific</td>
<td>32</td>
<td>C2 30B</td>
<td>0/28</td>
<td></td>
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<tr>
<td>Inoculation of neoplastic cells</td>
<td></td>
<td>C3 30B</td>
<td>0/14</td>
<td></td>
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<tr>
<td></td>
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<td>Ch3 30B</td>
<td>3/21</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ch4 30B</td>
<td>5/25</td>
<td></td>
</tr>
<tr>
<td>Inoculation of filtrate of homogenised neoplastic cells</td>
<td>78</td>
<td>C4 30B</td>
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<td>C5 30B</td>
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<td>0/24</td>
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<tr>
<td></td>
<td></td>
<td>Ch6 30B</td>
<td>0/21</td>
<td></td>
</tr>
<tr>
<td>Interspecific</td>
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<td>C6 30A</td>
<td>0/28</td>
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<tr>
<td>Co-habitation</td>
<td></td>
<td>C7 30A</td>
<td>0/30</td>
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<tr>
<td></td>
<td></td>
<td>Ch7 30A + 25N</td>
<td>0/28</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Ch8 30A + 25N</td>
<td>0/29</td>
<td></td>
</tr>
</tbody>
</table>

No case of DN was detected in either cockles inoculated with a filtrate of homogenised haemolymph cells from DN-affected cockles or in the corresponding controls (Table 1). This trial ran for 78 days and 27 cockles could not be diagnosed after removal from tanks because their tissues were too degraded.
Regarding the clams *R. philippinarum* and *V. corrugata* taken from the shellfish bed located in Sarrido, where cockle DN prevalence was high, none of the clams were found to be affected by DN.

The evolution of cumulative survival of individuals of each experimental group in each tank is shown in Figure 1. In the case of the intraspecific transmission trial by co-habitation, survival was significantly higher in the cockles that were diagnosed as healthy (N0) before the start of the trial than in those with light (N1) and heavy (N3) DN severity (Figure 2).

**Discussion**

Results of the co-habitation trial support the hypothesis that DN can be transmitted from...
Previous attempts of transmission of DN among cockles by cohabitation conducted in the field were unsuccessful (Twomey and Mulcahy, 1988a). The transmission of DN by cohabitation of diseased and healthy individuals was demonstrated in Mya arenaria (Brown, 1980; Mateo et al., 2015) and Mytilus edulis (Elston et al., 1988). Furthermore, the results suggest that the cancer could be transmitted by inoculation of neoplastic cells in healthy individuals. It might be possible that neoplastic cells detected in inoculated individuals were the inoculated cells themselves. Nevertheless, some of the inoculated cockles were diagnosed as having heavy DN severity, which could have only been achieved by the proliferation of neoplastic cells in the recipient individual. The experimental design did not allow determination of whether the neoplastic cells proliferating in the inoculated individuals were derived from the injected cells or they were transformed cells of the recipient cockles. Transmission of DN between cockles Cerastoderma edule via injection of hemolymph (whole or cell-fraction) had been performed by Twomey and Mulcahy (1988b) and Collins and Mulcahy (2003). However, DN transmission by inoculation of a filtrate of homogenised haemolymph cells from DN-affected individuals was not detected in our trial. Twomey and Mulcahy (1988b) also failed transmitting DN into healthy cockles by this procedure, whereas Collins and Mulcahy (2003) reported transmission in two out of five attempts with a similar protocol. Transmission of DN into clams M. arenaria was also achieved by adding tissue homogenates from neoplastic clams to the water of tanks holding healthy clams (McGladdery et al., 2001).

Transmissibility of DN and the occurrence of epidemic outbreaks justify consideration of this disease as infectious. What is the infectious agent

Figure 2. Evolution of the cumulative survival of the cockles in each class of disseminated neoplasia severity (N0–N3) through the intraspecific transmission trial by co-habitation, according to the diagnosis at the start. Different letters above severity classes in the legend denote significant differences.
responsible for DN in cockles and, in a broader sense, in molluscs? This study showed evidence of transmission with neoplastic cells, but attempts of DN transmission by inoculating a filtrate of homogenised haemolymph cells from diseased cockles, which could have allowed the inoculation of a hypothetical virus infecting neoplastic cells, failed. Detection of reverse transcriptase activity associated with DN has been interpreted as suggesting a retroviral aetiology for this disease in cockles (Romalde et al., 2007) and clams Polititapes aureus (= Venerupis aurea) (Manso et al., 2012) from Galicia. Other authors questioned whether detecting reverse transcriptase activity was enough to support retroviral aetiology of DN in molluscs, because that activity could be endogenous (AboElKahair et al., 2009a, 2009b, 2012). Siah et al. (2011) showed evidence of an endogenous retrotransposon involvement in DN of clams M. arenaria. Recently, a retrotransposon called “Steamer” has been characterised, expression of which is correlated with DN in clams M. arenaria (Arriagada et al., 2014). Therefore, evidence other than reverse transcriptase activity would be needed to support viral aetiology. Recent findings showing that the cancerous cells correspond to cell lineages with a genotype different from that of the normal cells of the affected individuals of M. arenaria (Metzger et al., 2015), M. trossulus, C. edule and P. aureus (Metzger et al., 2016) led to the conclusion that DN is transmitted horizontally between individuals as contagious cancer cells. Our results, including the transmission failure with a filtrate of homogenised haemolymph cells from DN-affected cockles, are also consistent with DN transmission between cockles as contagious cancer cells.

The significantly higher survival of cockles that were diagnosed as non-DN-affected before the start of the co-habitation trial than that of cockles with light and heavy DN would support the conclusion that DN can cause cockle mortality. Field studies support this statement (Díaz et al., 2016). Laboratory (Cooper et al., 1982) and field (Brousseau and Baglivo, 1991) experiments also showed higher mortality of DN-affected clams M. arenaria than that of non-affected ones.

No evidence of interspecific transmission of DN, from cockle to clams, was found in this study. Laboratory transmission attempted by co-habitation failed and no DN-affected clam was found in samples from the bed in Sarrido, where cockle DN is highly prevalent. Previous studies detected DN in clams R. decussatus from Galicia at very low prevalence (Villalba et al., 1995). Other attempts of interspecific DN transmission have also failed (Kent et al., 1991). However, there is evidence of cancerous cells proliferating in P. aureus derived from a different clam species, V. corrugata, which opens a new perspective on cross species transmission of cancer (Metzger et al., 2016).

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