Development of an experimental model for Anophryoides haemophila (Scuticociliatida: Orchitophryidae), a parasite of American lobster Homerus americanus

F. ATHANASSOPOULOU (Φ. ΑΘΑΝΑΣΟΠΟΥΛΟΥ), D. SPEARE, R. J. CAWTHORN, R. MacMILLAN, B. DESPRES

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**Development of an experimental model for Anophryoides haemophila** (Scuticociliatida: Orchitophryidae), a parasite of American lobster *Homarus americanus*.

Athanassopoulou F., Speare D., Cawthorn R.J., MacMillan R., Despres B.

ABSTRACT. The principal lesions due to the ciliate *Anophryoides haemophila* in experimentally infected lobsters are observed in gills and connective tissue from the 9th week post-infection. These lesions were not related to the inoculum of parasites and were consistent in all inoculum studies (2,000, 10,000 and 500,000 ciliates per lobster) and were present in both euthanized and dead lobsters. The higher inocula resulted only in higher numbers of parasites observed in sections of gills and in the connective tissue of more organs, with no penetration to the parenchyma of any organ. Furthermore, the highest inoculum of ciliates (500,000 per lobster) resulted in a shorter course of disease development and consequently, experimental lobsters died between 4th and 6th week post-infection. This was not observed at inocula of 2,000 and 10,000 ciliates per lobster, where mortality occurred 11-14 weeks post-infection.

**Keywords:** *Anophryoides haemophila*, American lobster, *Homarus americanus*, Scuticociliatida, experimental conditions.
INTRODUCTION

Ciliate parasites are generally considered as etiologic agents of important diseases of crustaceans (Morado & Small, 1995) and can cause post harvest losses of American lobster (Homarus americanus) up to 15% annually (Cawthorn, 1997). "Bumper car" disease is caused by an Anophryoides haemophila, a scuticociliate, recently described by Cawthorn et al. (1996). Previously studies of experimentally induced disease in lobsters are rare (Sherbourne & Bean, 1991).

Overall, there is a significant requirement for development of experimental models of studying health and infectious diseases in lobsters as this would facilitate the comparison and analysis of results among different researchers and to assess the impact of infectious diseases on both wild and captive crustacean populations. Recently, a long term project has started at the University of Prince Edward Island to establish laboratory health data and haematological parameters of healthy and diseased lobsters and to assess risk factors for lobsters in holding facilities.

The present study is part of this project to establish an experimental useful model for the study of "bumper car disease" in lobsters, in terms of sequential development of lesions arising from the ciliate infection in these standardized experimental conditions. Ultimately, this model could provide useful information related to disease prevention and effective treatment.

MATERIALS AND METHODS

Source and maintenance of lobsters

'Canner' lobsters (carapace length 65.1-80.9 mm, weight 190-400 g) were purchased during the spring lobster season period and transported to the Aquatic Animal Facility, Atlantic Veterinary College. Lobsters were held in a system of six tanks, 4770 L capacity, saltwater recirculation system that was equipped with both particle and biological filtration. Artificial seawater was prepared from Instant Ocean. Each tank housed 20 lobsters (all claws banded) in individual compartments in stacked trays. Water quality was monitored weekly for unionized ammonia (<0.01 mg/L), nitrate ion levels (<20.0 mg/L), nitrite ion (<0.1 mg/L), pH (range: 7.9-8.4), and salinity (range: 29-30 ppt), and continuously for temperature (2±1 °C). Lobsters were not fed during the experiments and the system was monitored daily for ill or dead lobsters. Lobsters were maintained in accordance with the Guidelines of the Canadian Council on Animal Care, on a 12h light:12h dark photoperiod. Lobsters used in both experiments were kept in similar experimental conditions. The haematolymph of ten lobsters of the stock intended for experimentation was examined for ciliate infection and gaffkaemia seven days prior to the initiation of the experiments.

Pilot study to assess inoculum level and sampling times

Twelve healthy lobsters were used for this experiment.
Lobsters were inoculated with 1,000, 10,000 and 500,000 ciliates per animal by intrahaemocoelic injection (three lobsters per inoculum). Haemolymph was removed at weekly intervals and examined for the presence of parasites by light microscopy. An equal number of lobsters was inoculated with sterile seawater and the lobsters were used as controls. Lobsters were not euthanized, but left to die and were sampled at that point for routine histology, immunohistochemistry and immunofluorescence.

Haemolymph was drawn at weekly intervals and examined under light microscopy and immunofluorescence for the presence of parasites.

**Source and maintenance of ciliates**

*Anophryoides haemophilia* originated from experimentally infected lobsters at Atlantic Veterinary College. Ciliates were subpassaged in 25 cm² culture flasks containing 10 ml of modified ATCC 1651 MA medium (Messick & Small, 1996). Ciliates were subpassaged to new flasks every 14 days by using 0.5 ml of established culture in 9.5 ml of fresh medium. Cultures were maintained at 5°C. Lobsters used in both experiments were inoculated with ciliates from the same culture.

**Ciliate counts prior to infection**

Established cultures of ciliates were placed in 15 ml centrifuge tubes and spun at 600 G for 5 min in refrigerated centrifuge. The resulting sediment was then removed, resuspended in 8 ml of artificial seawater and gently agitated. Counts of the ciliates were made by using an haemocytometer, after fixing the ciliates in neutral buffered formalin 10%. Appropriate dilutions were then performed in order to obtain the doses required for the experiments.

**Haemocytes and ciliate collection and counts**

Haemolymph was obtained by puncture of the ventral hemal sinus and ciliates were collected, using a 5 ml syringe filled with 4.5 ml of buffer (Cornick & Stewart, 1968) and 0.5 ml of haemolymph which was removed from each lobster. Counts were made by a haemocytometer.

**Histology**

Tissues removed from infected and uninfected lobsters included gills, hepatopancreas, muscles, heart, intestine, antennal gland, stomach and reproductive tissue, haemopoietic tissue, nerve ganglion and epidermis. Tissues were fixed in 1 part glutaraldehyde and 4 parts formalin for 60 min. Sections were dehydrated in an ethanol series and embedded in paraffin. 5 μm sections were cut, mounted on glass slides and stained with haematoxylin and eosin.

**Immunohistochemistry staining**

Immunoperoxidase staining of ciliates, using a monoclonal antibody (clone 16H2), was used (Cawthorn, unpublished data) to determine the presence of parasites in the organs of lobsters at early stages of the experimental
Πίνακας 1. Βιωσιμότητα, συνολικός αριθμός παρασίτων την ώρα του θανάτου και αποτελέσματα ανοσοφθορισμού και ανοσοπεροξειδάσης σε πειραματικά μολυσμένους αστακούς με διαφορετικές δόσεις εμβολιασμού με Anophryoides haemophila.

<table>
<thead>
<tr>
<th>Αριθμός παρασίτων που ενοφθαλάμτηκαν ανά αστακό</th>
<th>Εβδομάδες μέχρι το θάνατο</th>
<th>Αριθμός παρασίτων ανά ml αιμολέμφου (x10⁶)</th>
<th>Θετικό σε δοκιμασία</th>
<th>Θετικό στην αντίδραση ανοσοπεροξειδάσης</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>8-10</td>
<td>0,14 (10 εβδομ)</td>
<td>4 εβδομάδες</td>
<td>ΔΕ</td>
</tr>
<tr>
<td>10000</td>
<td>10-14</td>
<td>0,2 (10 εβδομ)</td>
<td>4 εβδομάδες</td>
<td>4 εβδομάδες</td>
</tr>
<tr>
<td>500000</td>
<td>4-6</td>
<td>0,9 (14 εβδομ)</td>
<td>1 εβδομάδα</td>
<td>1 εβδομάδα</td>
</tr>
</tbody>
</table>

C = μάρτυρας, M = μολυσμένων, ΔΕ = δεν έγινε

Table 1. Survival, total number of ciliates at time of death and immunofluorescence and immunoperoxidase results of experimentally infected lobsters with different inocula of Anophryoides haemophila.

<table>
<thead>
<tr>
<th>Number of ciliates inoculated per lobster</th>
<th>Number of weeks till death (weeks)</th>
<th>Number of ciliates per ml of haemolymph (x10⁶)</th>
<th>IFAT +ve</th>
<th>Immunoperoxidase +ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000</td>
<td>8-10</td>
<td>0,14 (10 w.)</td>
<td>4 weeks</td>
<td>ND</td>
</tr>
<tr>
<td>10,000</td>
<td>10-14</td>
<td>0,2 (10 w.)</td>
<td>4 weeks</td>
<td>4 weeks</td>
</tr>
<tr>
<td>500,000</td>
<td>4-6</td>
<td>0,9 (14 w.)</td>
<td>1 week</td>
<td>1 week</td>
</tr>
</tbody>
</table>

ND = not done, C = control, M = mortality, W = week

Χρώση ανοσοϊστοχημείας

Για να καθορισθεί η παρουσία παρασίτων στα οργάνα των αστακών σε πρώιμα στάδια των πειραματικών μολύνσεων χρησιμοποιήθηκε χρωστική ανοσοπεροξειδάση για βλεφαριδοφόρους παράσιτους, η οποία χρησιμοποιεί έναν κλόνο αντίσωμα (clone 16H2) (Cawthorn, unpublished data). Τμήματα ανοσοϊστοχημείας επικολλήθηκαν σε θετικά φορτηγέτες slides, χρησιμοποιώντας τη μέθοδο του Speare και των συνεργατών του (1998), για απόδοση του Loma salmonae.

Χιονίδια, έντερο, πρόσθιο αδένα, στόμαχο και ιστοί του γεννητικού, αιμοποιητικού ιστού, νευρικά γάγγλια και επιδερμίδα. Οι ιστοί μονιμοποιήθηκαν σε 1 μέρος γλουταρδειδής, 4 μέρη φορμαλινής, σύμφωνα με την μέθοδο McDowell & Trump (1976). Οι αστακοί θανατώθηκαν με εμβάπτιση σε βενζοκαϊνή (10% σε αιθανόλη), σε μια δόση 18 ml ανά λίτρο θαλασσινού νερού.

infections. Sections for immunochemistry were mounted on positively charged slides, using the method of Speare et al (1998) for Loma salmonae spores.

Immunofluorescence

10 μl of ciliate suspension was air dried on slides with 12 wells and acetone was fixed for 10 min. Slides were then stored at -20°C. Supernatants, from wells containing the clone, were tested by fluorescence antibody (FA). Slides were washed for 1 min in phosphate buffered saline (PBS) to remove salts from the seawater and 20 μl of supernatant was inoculated on each well for 20 min at room temperature (25°C). Slides were then washed with PBS and a second antibody goat antimouse IgG+M+A, labeled with fluorescein (1/100 dilution), was incubated for 20 min at room temperature in the dark, with Evans Blue as a counterstain. Slides were washed again with PBS and mounted with FA mounting fluid.
RESULTS

Clinical signs and necropsy

No clinical signs were observed during the experimental studies and lobsters were considered dead when completely non-responsive to external stimuli.

Pilot study to assess inoculum level and sampling times

The number of ciliates found in haemolymph at death, during infections with different inocula numbers, is shown in Table 1 & 2. Both immunofluorescence and immunoperoxidase testing of tissue sections revealed the presence of ciliates first in the gills (week 4) with most inocula, whereas for the highest inoculum the ciliates reacted positively in gills from the first week.

The ciliate counts in haemolymph are presented in Table 2. The ciliates were recognizable by light microscopy examination at week 5. In immunofluorescence testing in tissue sections, the parasite was first present in the gills at week 4 post-infection, only in the gills (Table 2). No lesions were observed until week 5 post-infection. However, the principal lesions were observed in gills and connective tissue from 9th week post-infection for the lower inocula and at week 4 for the 500,000 ciliate inoculum. These lesions were not related to the inoculum of parasites and were present in all inoculum studies. Higher doses resulted only in higher numbers of parasites observed in sections of gills and in the connective tissue of more organs, with no penetration to the parenchyma of any organ.

Furthermore, the highest inoculum of ciliates (ie. 500,000 per lobster) resulted in a shorter course of disease development: this was not observed at inoculums of 1,000, 2000 and 10,000 per lobster.

The most characteristic lesions, causing severe pathology in experimentally infected animals, typically occurred at later stages of infection, in the gills and the connective tissue, and are described in detail in another paper (Athanassopoulou et al., unpublished data).

DISCUSSION

Reports of ciliate infections in wild populations of crustaceans are normally associated with molting periods, localized physical parameters areas (Messick & Small, 1996) and puncture wounds (Sherbourne & Bean, 1991; Morado & Small, 1995). Therefore, it is necessary to make a prediction of an accurate parasite inoculum that initiates infections in nature and to establish a comparative experimental model. Infections in crabs are not useful experimental models. Infections in crabs are not useful experimental stages in lobsters, because their development of ciliate-induced disease has very different physical parameters areas (Messick & Small, 1996) and puncture wounds (Sherbourne & Bean, 1991; Morado & Small, 1995). Therefore, it is necessary to make a prediction of an accurate parasite inoculum that initiates infections in nature and to establish a comparative experimental model. Infections in crabs are not useful experimental models. Infections in crabs are not useful experimental stages in lobsters, because the development of ciliate-induced disease has very different time scale in these hosts (Bang et al., 1972; Armstrong et al., 1981). All naturally reported infections in American lobsters have a comparatively short course of disease, with mortalities occurring at week 6 or 8 post-infection (Aitken & Waddy, 1986).

There are no previous reports of experimental induction
Πίνακας 2. Βιωσιμότητα, συνολικός αριθμός παρασίτων Anophryoides haemophila την ώρα του θανάτου και αποτελέσματα ανοσοφθορισμού και ανοσοπεροξειδάσης σε πειραματικά μολυσμένους αστακούς με 2000 παράσιτα ανά αστακό.

<table>
<thead>
<tr>
<th>Αριθμός παρασίτων που ενοφθαλμίστηκαν ανά αστακό</th>
<th>Εβδομάδες μέχρι το θάνατο</th>
<th>Μέσος αριθμός (χ) παρασίτων ανά ml αιμολέμφου (χ10³) (SE)</th>
<th>Θετικό σε δοκιμασία IFAT</th>
<th>Θετικό στην αντίδραση ανοσοπεροξειδάσης</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>4-12 εβδομάδες</td>
<td>E (N=3) - C (N=3) - M (N=3)</td>
<td>4 εβδομάδες (βράγχια)</td>
<td>4 εβδομάδες (βράγχια)</td>
</tr>
<tr>
<td></td>
<td>6 εβδομάδες</td>
<td>0 0 0,09 (+4,1)</td>
<td>4 εβδομάδες (βράγχια)</td>
<td>4 εβδομάδες (βράγχια)</td>
</tr>
<tr>
<td></td>
<td>7 εβδομάδες</td>
<td>0 0 0,07 (+3,5)</td>
<td>4 εβδομάδες (βράγχια)</td>
<td>4 εβδομάδες (βράγχια)</td>
</tr>
<tr>
<td></td>
<td>9 εβδομάδες</td>
<td>0,2 0 0,2 (+4,3)</td>
<td>4 εβδομάδες (βράγχια)</td>
<td>4 εβδομάδες (βράγχια)</td>
</tr>
<tr>
<td></td>
<td>11 εβδομάδες</td>
<td>2 0 0</td>
<td>4 εβδομάδες (βράγχια)</td>
<td>4 εβδομάδες (βράγχια)</td>
</tr>
<tr>
<td></td>
<td>12 εβδομάδες</td>
<td>0 0 3 (+1,1)</td>
<td>4 εβδομάδες (βράγχια)</td>
<td>4 εβδομάδες (βράγχια)</td>
</tr>
</tbody>
</table>

E = ευθανασία, M = μολυσμένων κατά την ημέρα θανάτου, C = μάρτυρες

| ΠΕΡΙΟΔΙΚΟ ΤΗΣ ΕΛΛΗΝΙΚΗΣ ΚΤΗΝΙΑΤΡΙΚΗΣ ΕΤΑΙΡΕΙΑΣ 2003,54(3) | JOURNAL OF THE HELLENIC VETERINARY MEDICAL SOCIETY 2003,54(3) |

Table 2. Survival, total number of Anophryoides haemophila at time of death and immunofluorescence and immunoperoxidase results of experimentally infected lobsters with 2,000 ciliates per lobster.

<table>
<thead>
<tr>
<th>Number of ciliates inoculated per lobster</th>
<th>Number of weeks till death (weeks)</th>
<th>Number of ciliates per ml of haemolymph (χ10³) (SE)</th>
<th>IFAT +ve</th>
<th>Immunoperoxidase +ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>4-12 weeks</td>
<td>E (n=3) - C (n=3) - M (n=3)</td>
<td>4 weeks (gills)</td>
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<td>0 0 0,09 (+4,1)</td>
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</tr>
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<td></td>
<td>12 weeks</td>
<td>0 0 3 (+1,1)</td>
<td>4 weeks (gills)</td>
<td>4 weeks (gills)</td>
</tr>
</tbody>
</table>

E = euthanised, M= mortality, C = control
The results of a further experiment (Athanassopoulou et al., unpublished data) showed that the "bumper car" disease under experimental conditions appears to develop in two phases. A first period (weeks 1-4 post-infection), where no parasites can be detected in the lymph and a second period (weeks 5 onwards), when parasites appear in the lymph and increase steadily to very high numbers. This pattern is highly correlated with the sudden decrease in haemocyte numbers in the haemolymph, observed at week 5 from infectivity studies with similar inocula, run in parallel to these experiments (Cawthorn et al, unpublished data). This two-phase development of the infection is documented by the histopathological changes and the immunofluorescence and immunoperoxidase observations.

Consequently, because the present study showed that the lesions were not dose related, we suggest that an inoculum as low as 2,000 ciliates per animal allows a gradual and satisfactory study of this disease in lobsters.

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500,000 ciliates/lobster was used.


