

NOTE

Response of American lobsters *Homarus americanus* to infection with a field isolate of *Aerococcus viridans* var. *homari* (gaffkemia): survival and haematology

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ABSTRACT: American lobsters *Homarus americanus* were inoculated with a field isolate of the Gram-positive bacterium *Aerococcus viridans* var. *homari*, causative agent of gaffkemia, at 1×10^6 , 1×10^4 or 1×10^2 colony forming units (CFU) kg^{-1} or with sterile 3% NaCl and maintained at 10 or 15°C until they died or were euthanised. Progression of disease in individual animals was monitored daily by total haemocyte count (THC) and haemolymph culture. Post-mortem examinations were performed on all lobsters. Effects of both ambient temperature and infective dose on survival time were observed. Marked bacteraemia occurred in all mortalities. Haemocytopenia (THC $< 10 \times 10^9$ cells l^{-1}) preceded death in most, but not all, mortalities.

KEY WORDS: Lobster · *Homarus americanus* · Gaffkemia · *Aerococcus viridans* var. *homari*

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INTRODUCTION

Gaffkemia, a generally fatal systemic bacterial disease of the American lobster *Homarus americanus* caused by the Gram positive coccus *Aerococcus viridans* var. *homari*, has been extensively investigated and reviewed (Stewart 1980, 1993). It is considered to be a temperature-dependent disease (Cornick & Stewart 1968, Stewart et al. 1969b, Stewart 1980, 1993). Latent infections (lobster-passaged *A. viridans*, Rabin's strain) remain essentially dormant when lobsters are held at 1°C, while warming to 3°C results in classical disease (Stewart et al. 1969b). Infections occur when bacteria gain entry through breaks in the exoskeleton. Eventually, large numbers of cocci, often arranged in chains or tetrads, are visible in the haemolymph. Total

haemocyte counts (THCs) are usually very low in the terminal stages (Stewart et al. 1969a, 1983). Mass mortality, and consequently significant economic loss, can result when outbreaks occur in lobster pounds (Hästein & Roald 1977, Menard & Myrand 1987).

A small-scale study was designed to examine the effects of infective dose and ambient temperature on lobster survival times and THCs using a field isolate of *Aerococcus viridans*. Serial sampling permitted monitoring of disease progression in individual animals.

MATERIALS AND METHODS

Animals and housing. For the acclimation period, 42 hard-shelled, intermoult, male and female lobsters

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were obtained from a commercial dryland pound (Clearwater Fine Foods). Lobsters were kept individually in compartmentalised plastic trays while acclimated for 4 wk in a recirculating water (artificial seawater [ASW] Instant Ocean®, Aquarium Systems) system maintained between 1.5 and 2.5°C. Lighting was set at a 14 h low light (<3 lux):10 h dark cycle. Lobsters were not fed during the acclimation period.

For the experimental period, the lobsters were kept individually in compartmentalised plastic trays in recirculating ASW tank systems at either 10 or 15°C ($\pm 1.5^\circ\text{C}$). Additional ASW was added if ammonia levels exceeded 0.3 ppm. Room lighting was maintained at a 14 h low light (<3 lux):10 h dark cycle. Lobsters were not fed during the experimental period.

Inoculum preparation. A lyophilised culture of an *Aerococcus viridans* Type 3 (api 20 Strep, bio-Mérieux Canada) was used. It was recovered from a lobster submitted to the Atlantic Veterinary College Diagnostic Laboratory as part of an investigation of a gaffkemia outbreak at a commercial lobster pound. A thawed aliquot of a first passage 48 h subculture stored at -80°C in 2% skim milk culture broth (Bacto® skim milk, DIFCO Laboratories) was grown on sheep blood (5%) agar (BA) (Oxoid) at 28°C for 24 h and the colonies were used to inoculate 25 ml of sterile trypticase soy broth (TSB). Broth cultures were incubated overnight at 28°C and the optical density (OD_{420}) was read at 420 nm (Spectronic 20D, Milton Roy Company). The suspension was washed twice with sterile 3% NaCl. An aliquot of the saline suspension was serially diluted to obtain inoculum doses of 1×10^6 , 1×10^4 and 1×10^2 colony forming units (CFU) kg^{-1} lobster for the high (HD), intermediate (ID) and low dose (LD) groups, respectively. Colony counts were performed on 25 μl aliquots of the serial dilutions, cultured on BA at 28°C after 48 h of incubation.

Direct haemolymph culture. To detect bacteraemia, sheep BA (5%) plates were inoculated with 0.2 to 0.4 ml of aseptically collected haemolymph, incubated at 28°C, and examined at 24, 48 and 72 h. The morphology, Gram stain and catalase activity of all colonies were recorded (Tortura et al. 1995).

Challenge protocol. After 3 d of acclimation at 2°C, haemolymph samples were collected from the ventral abdominal sinus at the first or second abdominal segment (20G needle) after surface disinfection with 70% isopropyl alcohol. For determination of THCs (Neubauer haemocytometer, Dynatech), 0.5 ml of haemolymph was collected into a 5.0 ml syringe containing 4.5 ml of anticoagulant (28.4 g l^{-1} NaCl, 8.7 g l^{-1} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.5 g l^{-1} $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.5 g l^{-1} $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.7 g l^{-1} KCl, 0.5 ml Tween 80, 1.25 ml l^{-1} stock [37 to 40%] formaldehyde solution, pH 7.6)

(Stewart et al. 1967). Undiluted haemolymph was examined for the presence of either the ciliate protozoan *Anophryoides haemophila* or *Aerococcus viridans* by brightfield light microscopy (BLM). To detect low level ciliate infections, 4 ml of ciliate culture medium (Modified ATCC 1651 Marine Axenic Medium for Marine Ciliate Culture) were inoculated with 0.5 ml of haemolymph, incubated at 4°C for 1 wk, and then examined by BLM (Messick & Small 1996).

After 4 wk of acclimation at 2°C, haemolymph was collected to establish baseline (Week 0) values at 2°C for THC and BLM examination. In addition, duplicate phenyl ethyl alcohol (PEA) broth tubes were inoculated with 0.5 ml of haemolymph and incubated at 28°C for 96 h (Stewart et al. 1966). Twenty-nine lobsters with a $\text{THC} > 10 \times 10^9 \text{ cells l}^{-1}$, negative BLM examination and negative cultures were selected and randomly assigned to the 10°C ($n = 15$) or 15°C ($n = 14$) experimental tanks. Prior to the addition of lobsters, each tank was swabbed and the samples cultured (BA at 28°C for 72 h) to check for the presence of *Aerococcus viridans*-like organisms in the system. Haemolymph samples were collected again at 1 and 2 wk for THC, PEA broth inoculation and BLM.

After 2 wk at 10 or 15°C, lobsters (body weights from 491 to 628 g) with a $\text{THC} > 10 \times 10^9 \text{ cells l}^{-1}$ and negative PEA and BLM were randomly assigned to the HD, ID, LD treatment groups or the control group (sterile 3% NaCl) within their respective tanks. There were 9 male and 3 female lobsters in the 10°C tank and 8 male and 4 female lobsters in the 15°C tank. Lobsters were inoculated (0.16 to 0.20 ml per lobster) by injection into the ventral abdominal sinus at the first abdominal segment. Haemolymph samples were collected daily from Day 1 post-inoculation (D1 p.i.). Samples were processed for THC, BA plate inoculation and BLM.

Daily sampling continued through to D20 p.i., at which point all lobsters in the HD and ID groups had died. The remaining 12 lobsters were euthanised by KCl injection (100 mg KCl g^{-1} lobster) at D21 p.i. (Battison et al. 2000). The walls and biofilters from each tank were swabbed and the samples cultured to check for the presence of *Aerococcus viridans*.

Post-mortem examinations were performed and tissues (heart, hepatopancreas, gill, gut, antennal gland, gonad, abdominal [tail] muscle and ventral nerve cord) were collected, placed in a glutaraldehyde (1%)-formalin (4%)-ASW fixative and embedded in paraffin (National Oceanic and Atmospheric Administration 1983). Sections of 5 μm were cut, routinely processed and stained with haematoxylin and eosin for histological examination.

All statistical analyses were performed using the Minitab release 11.12 software package. Non-parametric testing procedures were used due to the small sample sizes ($n < 30$). A p-value of < 0.05 was considered significant unless Bonferroni adjustments of significance level were required.

RESULTS

The data and statistical analyses presented are from the 24 lobsters that were followed for the entire experiment.

Acclimation at 10 and 15°C

Aerococcus viridans was not identified in cultures from the experimental tanks. Transient (1 to 2 d) problems with ammonia levels and temperature regulation were corrected within the first week. Two lobsters died within the first 8 d of acclimation, each was positive for *A. viridans* on PEA culture at either Week 0 (10°C) or Week 1 (15°C).

THCs in both systems tended to increase after transfer to warmer temperatures (Table 1). There was a significant difference among median THCs by 'week' at 10°C ($p = 0.001$, Friedman test, blocking on lobster) and between tanks after 2 wk ($p = 0.0011$, Mann-Whitney test at $p < 0.017$, Bonferroni adjusted significance) (Table 1). The percent changes (% change = $[\text{THC}_{\text{week } x} / \text{THC}_{\text{week } 0}] \times 100$) in median THC from 0 wk were greater in lobsters held at 10°C than at 15°C at Week 1 and Week 2 ($p = 0.016$ and $p = 0.0019$, respectively, Mann-Whitney test at $p < 0.025$, Bonferroni adjusted significance) as were the absolute changes ($p = 0.013$ and $p = 0.001$, respectively, Mann-Whitney test at $p < 0.025$, Bonferroni adjusted significance).

Table 1. *Homarus americanus*. Total haemocyte counts (THCs), determined using a Neubauer haemocytometer) at Week 0 at 2°C and during acclimation at 10 or 15°C ($n = 12$ for both temperatures), and percent of THCs at Week 0, by tank. Values are range (median). Column medians significantly different at * $p < 0.017$ or ** $p < 0.025$, Mann Whitney test, Bonferroni adjusted significance levels

Tank	THC ($\times 10^9$ cells l^{-1})			Percent of THC (%)	
	Week 0	Week 1	Week 2	Week 1	Week 2
10°C ^a	9.9–20.9 (13.3)	16.2–35.2 (25.4)	18.1–33.1 (25.1)*	107.2–337.4 (197.1)**	93.3–256.6 (194.0)**
15°C	10.4–20.9 (13.8) ^b	12.8–30.9 (18.2)	10.1–26.1 (16.1)*	93.4–214.6 (122.6)** ^b	78.26–185.1 (115.3)** ^b

^aSignificant difference in absolute THC by week within the 10°C tank (Friedman test, $p = 0.001$, blocking on lobster)
^bOne sample clotted; $n = 11$

Post-inoculation period

Colony growth in the 25 μl aliquots of the bacterial suspension indicated that HD, ID and LD group lobsters had received inoculation doses of 9×10^5 , 9×10^3 and 9×10^1 CFU kg^{-1} , respectively.

Survival times ranged from 4 to 21 d (Table 2, Fig. 1). Lobsters euthanised at 21 d (LD and control groups) were treated as censored data and assigned survival times of 21 d for the purposes of data presentation and statistical evaluation. The 95% confidence intervals (CIs) for the HD and LD groups were calculated using the pooled standard deviation and 8 degrees of freedom. The CIs did not overlap, indicating effects of both temperature and infective dose on survival (Fig. 1). Visual inspection of the data for the LD groups shows that survival times from the HD and ID groups differ (Fig. 1).

Aerococcus viridans infection was first detected by haemolymph culture. Bacterial growth was initially light but became heavier as the infections progressed. All lobsters in the HD-10°C group were transiently positive (few colonies) on D1 p.i., for 1 or 2 d, then consistently positive from D4 or D5 p.i. One lobster in the HD-15°C group was persistently bacteraemic from D1 p.i., while the other 2 were positive from D2 p.i. Lobsters in the ID-15°C group were persistently bacteraemic from D5 or D6 p.i. Lobsters in the ID-10°C group were first positive on D7, D9, or D13 p.i. Two of the lobsters in the LD-10°C group each had 2 d of low bacterial growth (1 to 4 colonies) starting on D15 and D16 p.i. A lobster in the LD-15°C group was intermittently positive (1 to 4 colonies) after D5 p.i.; light growth was present on D20 p.i. All control group lobsters were transiently (1 to 2 d) positive for *A. viridans* (few colonies only) from D11 to D16 p.i.

Changes in THCs in the post-inoculation period varied between groups (Fig. 2). Initial transient increases were observed for most lobsters in the HD and ID groups, followed by relatively stable THCs until 2 to 4 d prior to death when they decreased to $< 10 \times 10^9 \text{ l}^{-1}$ (Fig. 2a,b). Notable exceptions were 2 ID-10°C lobsters and 1 ID-15°C lobster. No consistent trends in THC were observed in the LD groups (Fig. 2c). Inoculation with sterile 3% NaCl generally had little effect at either temperature (Fig. 2d). Transient episodes of low level bacteraemia in the LD and control groups were not associated with consistent changes in the THCs.

Table 2. *Homarus americanus*. Survival times (d) of lobsters inoculated with *Aerococcus viridans* (3 dose levels) or sterile 3% NaCl (control group) and maintained at 2 temperatures (n = 3 for all dose \times temperature combinations). CFU = colony forming units

Temp	1×10^6 CFU kg $^{-1}$	1×10^4 CFU kg $^{-1}$	1×10^2 CFU kg $^{-1}$	3% NaCl
10°C	11, 11, 13	17, 17, 19	21 ^a	21 ^a
15°C	4, 5, 5	8, 8, 8	21 ^a	21 ^a

^aLobsters were euthanised

Table 3. *Homarus americanus*. Summary of major gross and histologic lesions in lobsters inoculated with *Aerococcus viridans* (3 dose levels) or sterile 3% NaCl (control group) and maintained at 2 temperatures. Results reported as the number of lobsters with the lesion/number of lobsters where the observation was recorded or tissue was available (n = 3 for all dose and temperature combinations). CFU = colony forming units

	1×10^6 CFU kg $^{-1}$		1×10^4 CFU kg $^{-1}$		1×10^2 CFU kg $^{-1}$		3% NaCl	
	10°C	15°C	10°C	15°C	10°C	15°C	10°C	15°C
Gross								
Antennal gland	3/3	2/2	1/3	2/3	0/3	1/3	0/3	1/3
Histologic								
Antennal gland	3/3	3/3	1/3	3/3	0/2	1/3	0/3	1/3
Hepatopancreas	3/3	3/3	3/3	3/3	0/3	1/3	0/3	0/3

Gross pathology

Gross lesions associated with gaffkemia were limited to small white, miliary, diffuse to coalescing foci in the antennal glands (Table 3). Very mild lesions were present in the intermittently bacteraemic LD-15°C lobster and one 15°C control-group lobster. A second 15°C control-group lobster had multiple, large (2 to 4 mm diameter), firm, dark, solid nodules throughout the hepatopancreas.

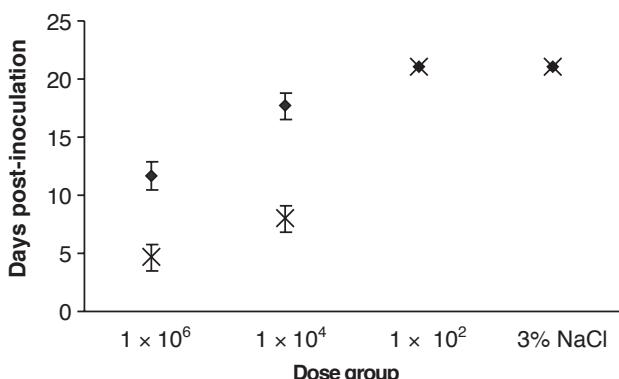


Fig. 1. *Homarus americanus*. Mean survival (d) and 95% confidence intervals for lobsters maintained at (♦) 10°C or (x) 15°C and inoculated with *Aerococcus viridans* at 1×10^6 or 1×10^4 colony forming units (CFU) kg $^{-1}$. Lobsters in the 1×10^2 CFU kg $^{-1}$ and sterile 3% NaCl control groups were euthanised on Day 21 and are included in the graph for comparative purposes

Histopathology

Post-mortem (up to 19 h) overgrowth of bacteria and autolytic changes were extensive in mortalities. A systemic bacterial infection was evidenced by the presence of haemocyte aggregates in the vascular spaces of most tissues examined.

Areas occupied by fixed phagocytes in the hepatopancreas were replaced by ballooned cells containing cocci with varying degrees of mixed haemocyte infiltrates with some organised aggregates containing eosinophilic to golden pigment (variably positive argentaffin reaction, Fontana-Masson stain). Lesions were present in all mortalities and in the LD-15°C bacteraemic lobster, but not other LD or control-group lobsters. The dark nodules observed grossly in the hepatopancreas of one 15°C control-group lobster were chronic, non-septic, inflammatory nodules with necrotic cores.

Inflammatory lesions in the antennal gland corresponded to the miliary foci observed grossly. These consisted of multifocal to coalescing nodular aggregates of haemocytes which often contained numerous cocci and eosinophilic to golden hyaline material. The labyrinth region of the antennal gland was more frequently affected, particularly in the HD-15°C group. Overall lesion severity was also greater in this group. Coccidi were not identified in the antennal gland in the 15°C control-group lobster with gross lesions. No histologic changes were noted in the 2 ID-10°C lobsters which had THCs above 20×10^9 l $^{-1}$ at the time of death.

Haematopoietic tissue (HPT) lobules (available in 3/12 lobsters held at 10°C and 3/12 lobsters held at 15°C) were cellular and dominated by immature haematopoietic cells. Mitotic figures were observed only in HPT from lobsters held at 15°C.

DISCUSSION

The marked ante-mortem bacteraemia, distribution and appearance of gross and histologic lesions, and haemocytopenia parallel findings of other gaffkemia studies. However, the apparent effect of infective dose on survival time does not (Johnson et al. 1981, Stewart 1980, Stewart et al. 1983). A previous study using a continuous flow system and inoculum levels from 1×10^1 to 1×10^9 organisms kg $^{-1}$ (Rabin's strain, lobster passaged) showed variation in mean time to death

(MTD) among different dose levels, but the differences were not statistically significant (Cornick & Stewart 1968).

Survival times of 4 to 5 d for lobsters in the HD-15°C group in the current experimental model were shorter

than other studies using lobster-passaged Rabin's strain of *Aerococcus viridans* at a similar dose and temperature in continuous flow systems. Stewart et al. (1969b) reported a MTD of 12 d at a dose of 5×10^6 organisms kg^{-1} . Johnson et al. (1981) recorded survival

times ranging from 8 to ≥ 14 d for lobsters inoculated with 1×10^6 organisms kg^{-1} . Direct comparison of these models is not appropriate. Inherent differences in the bacterial isolates, host factors and experimental system design, combined with the effects of stress from abrupt temperature changes, daily handling and haemolymph collection in the current study likely contributed to the different outcomes. The stresses experienced in the current study may, in part, mimic some of the conditions lobsters are subjected to as they are caught, transported and delivered to commercial holding pounds—situations which involve multiple handling events, abrupt temperature changes and, often, minor trauma.

Inability to identify the organism or classic histologic lesions in tissues from LD and control-group lobsters which had a mild, transient bacteraemia could indicate that bacterial concentrations were either too low to be detected by these techniques, or that the lobsters had cleared the infection. Although uncommon, lobsters have been reported to survive experimental and naturally acquired infections with *Aerococcus viridans* (Stewart et al. 1966, Cornick & Stewart 1968). Culture of hepatopancreatic tissue, an early site of bacterial localisation, or an extended experimental period may have helped to differentiate between these possibilities. One LD-15°C lobster ultimately developed a significant bacteraemia by D20 p.i. and had gross and histologic lesions of gaffkemia.

Infection by environmental bacteria likely accounted for the transient, low level *Aerococcus viridans* bacteraemia detected in all control-group lobsters. Multiple puncture wounds associated with sample collection could have provided portals of entry for the bacteria released into the tank from infected lobsters. Activation of latent, endogenous infections or surface contamination during sampling are considered less likely. The mild, transient bacteraemia detected in 2 lobsters in the LD-10°C group may be characteristic of gaffkemia at this combination of infective dose and temperature or also represent environmental infections.

While the above findings point out a deficiency in the experimental tank system, i.e. that repeated infection was possible, potentially shortening survival times of lobsters held for

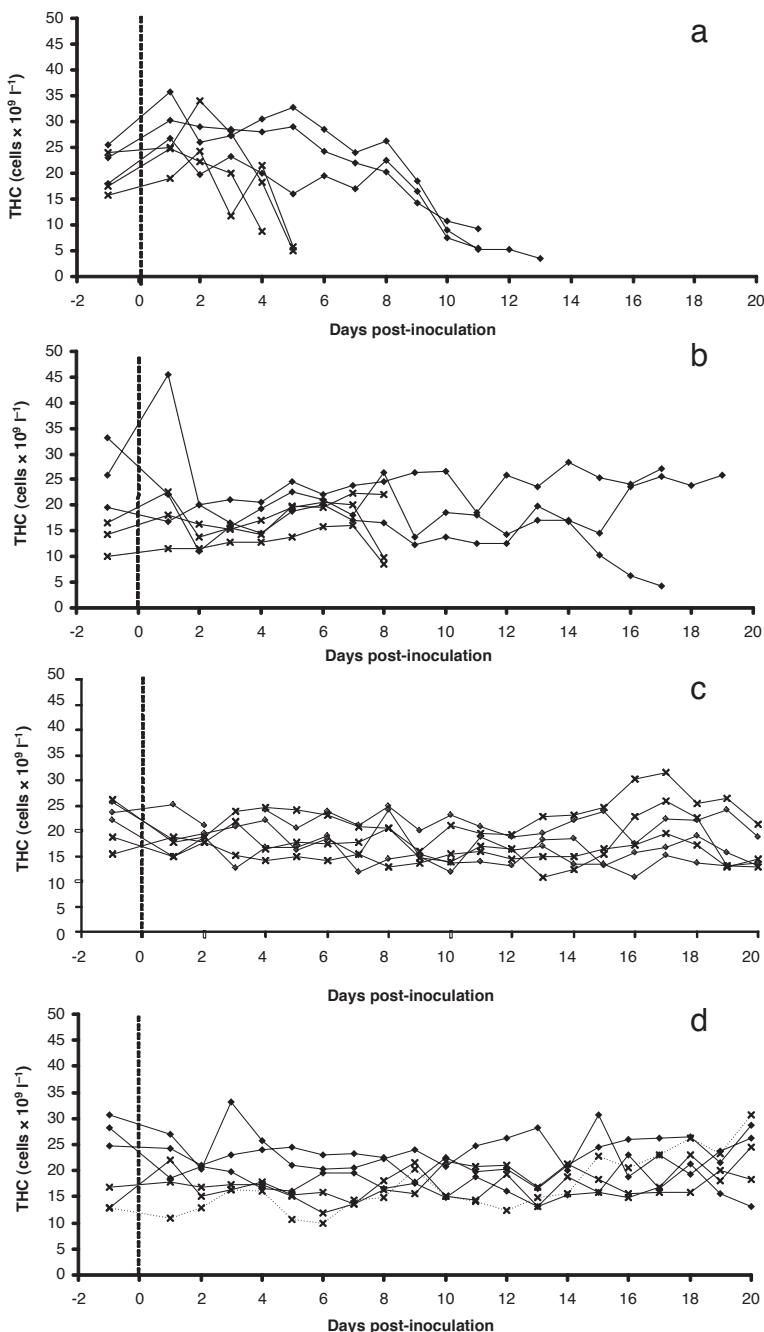


Fig. 2. *Homarus americanus*. Total haemocyte count (THC $\times 10^9$ cells l^{-1}) in lobsters inoculated on Day 0 with (a) 1×10^6 colony forming units (CFU) kg^{-1} , (b) 1×10^4 CFU kg^{-1} , (c) 1×10^2 CFU kg^{-1} of *Aerococcus viridans* or (d) sterile 3% NaCl (control). Lobsters were maintained at (◆) 10°C or (x) 15°C. Samples were collected until the lobsters died (1×10^6 and 1×10^4 CFU kg^{-1} groups) or were euthanised on Day 21 post-inoculation (1×10^2 CFU kg^{-1} and control groups)

longer periods, it did not adversely affect interpretation of the survival data. Significant differences in survival associated with both infective dose and temperature were observed.

Studies of *in vitro* phagocytosis by *Homarus americanus* haemocytes have demonstrated a temperature effect. Onset of phagocytosis (mixed haemocyte population) of opsonised sheep erythrocytes was similar at 10 and 15°C (20 min) although the percentage of haemocytes containing erythrocytes was slightly lower at 10°C (Paterson & Stewart 1974). Phagocytosis was delayed (60 min) at 4°C (Paterson & Stewart 1974). Phagocytosis (mixed haemocyte population) of an avirulent strain of *Aerococcus viridans* (MK-3) decreased at temperatures above 22°C compared to 16°C (Steenbergen et al. 1978). Initial transient bacteraemias in the HD-10°C lobsters may represent less effective clearance of the inoculum at the lower temperature. Effects of temperature on phagocytosis or bacteriocidal activity of the fixed phagocytes of the hepatopancreas have not been reported.

The overall pattern in the THCs, most notable in the HD groups, is consistent with other reports of gaffkemia and can be interpreted in terms of an inflammatory response (Stewart 1980, Stewart et al. 1983, Jain 1993). Mobilisation in response to the pathogen results in an initial increase in THC; however, as tissue demands for haemocytes exceed the supply, THCs decrease.

Haemocytopenia has been reported for *Homarus americanus* in the terminal phases of gaffkemia (Stewart et al. 1969a, Stewart 1980). The ability, or inability, of the HPT to adequately compensate for the increased consumption of haemocytes at the 2 temperatures was difficult to assess in this study, as HPT was only available for examination in 6 lobsters. Detection of mitotic figures only in the 15°C groups may indicate that temperature influences the activity of HPT in *H. americanus*, as has been observed in *Callinectes sapidus* (Johnson 1980).

Survival time of lobsters infected with a field isolate of *Aerococcus viridans* in this experimental system appeared to be related to both ambient temperature and infective dose. These results suggest that multiple variables, including the effects and inter-relationships of bacterial isolate, infective dose, ambient temperature, host factors and experimental system design, affect survival times and alterations in THCs.

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