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Febrile Response and Activity in the Crayfish, *Pacifasticus leniusculus trowbridgii*

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Title: Febrile Response and Activity in the Crayfish
       Pacifasticus leniusculus trowbridgii

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Leonard Simpson, Chairman

Richard B. Forbes

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     Poikilothermic and endothermic animals demonstrate
febrile response to infection with bacteria or to injection
with endogenous pyrogen extract of Prostaglandin E_1. Febrile
response is measured in endotherms as a relative change in
metabolically achieved body temperature and in poikilotherms

as an increase in selected temperatures relative to previously established preferred temperatures. Final preferendum change with environmental factors or associated physiological states.

Crayfish _Pacifasticus_ _leniusculus_ _trowbridgii_ were injected with 0.2 ml suspension of alcohol-killed gram-negative _Aeromonas_ _hydrophila_ bacteria (6×10^7) in 0.9% saline. Injections were into the coxopodite of the cheliped. The crayfish were monitored in an aquatic thermal gradient by means of a thermocouple attached to the carapace. The gradient range was from 9° to 26° C.

Temperatures were recorded and averaged for five minute time periods over two eight hour cycles, dark and light periods. Median values per animal per test run were used to define central tendency per animal. These values were used as data points to establish a treatment group mean or preferendum temperature. Comparsion recordings were completed with an uninfected group (baseline) and a group receiving 0.9% sterile pyrogen-free saline (control). Activity values or units were compared between groups. Activity was defined in terms of changes in temperature selection greater than 1.5^o C and in terms of rising or falling temperature values greater than 1.5^o C. Range values, 95% minimum and maximum, were also compared between groups.

Mean temperatures were not statistically different for any treatment group. Data representing activity values, range selection and behavior of the infected nocturnal - treatment crayfish were statistically different from the nocturnal treatment control and baseline data but were similar to all diurnal treatment data. Infected crayfish demonstrated reduced activity and narrower range preferences during nocturnal treatment periods.

FEBRILE RESPONSE AND ACTIVITY IN THE CRAYFISH

Pacifasticus leniusculus trowbridgii

by

KENNETH A. FLETCHER

A thesis submitted in partial fulfillment of the

requirements for the degree of

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in
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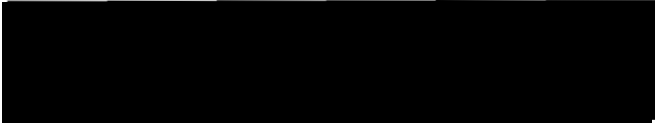
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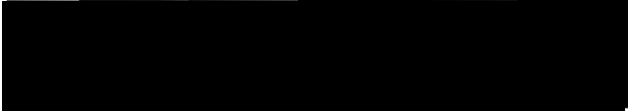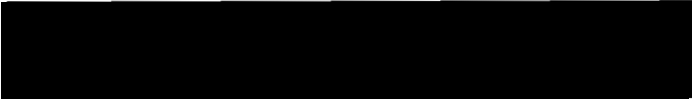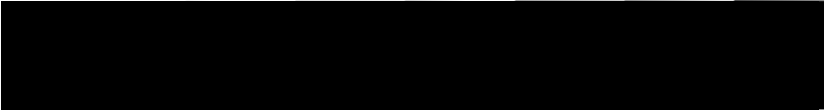LIST OF FIGURES

CHAPTER I

INTRODUCTION

Questions Examined in This Study

Fever induction due to infection has been demonstrated
in various species including turtles (Terrapene carolina and
Chrysemys picta, Monagas & Gatten, 1982), lizards
(Dipsosaurus dorsalis, Vaughn et al., 1974), frogs (Hyla
cinerea, Kluger, 1976) and salamanders (Necturus maculosus,
Hutchinson, 1981), goldfish (Carassius auratus, Covert &
Reynolds, 1977), and crayfish (Cambarus bartoni, Casterlin
& Reynolds, 1977). Febrile induction is accomplished
experimentally by injection of endogenous pyrogens from
crude or purified extract of Prostaglandin E_1 (PGE_1) or by
injecting live or attenuated bacteria. Typical bacterial
agents utilized are gram-negative bacteria such as Aeromonas
hydrophila, a bacillus found to be pyrogenic in vertebrates
and invertebrates (Casterlin & Reynolds, 1977; Monagas &
Gatten, 1982; Kluger et al., 1975; Tocco et al., 1983). In
endotherms, febrile response is measured as a relative
change in metabolically achieved body temperature. In
poikilotherms, febrile response or behavioral fever is
defined as an increase in selected temperatures relative to
previously established preferred temperatures (Reynolds,

1979). Terrestrial and aquatic vertebrates and invertebrate poikilothermic species have been shown to possess distinct thermal range preferences (Crawshaw, 1974; Mathur et al., 1982; Mathur & Silver, 1980; McCauley, 1977; Richards et al., 1977; Stauffer et al., 1975).

Fry (1947) defined the final thermal preferendum as the temperature "around which all individuals will congregate regardless of their thermal experience prior to placement in the gradient" and as "that temperature at which the preferred temperature is equal to the acclimation temperature". Final preferendum may change with environmental factors such as shelter and cover (Loring & Hill, 1976), season (Abrahmson & Goldmann, 1970); Momot & Gowing, 1972), photoperiods or circadian cycle (Crawshaw, 1974; Mason, 1963), predation (Stein, 1977), and water depth (Taylor, 1983). Associated physiological states such as sex or maturational state (Momot & Gowing, 1972), nutritional or trophic needs (Mason, 1963), and health (Casterlin & Reynolds, 1977,1978; Monagas & Gatten, 1982) also affect selection of a preferendum temperature. Heavy metals, chlorine and extreme pH or salinity alter temperature selection in crayfish (Crawshaw et al., 1980).

Activity and/or locomotor ability and availability of temperature ranges or gradients are critical parameters for selection of temperature preferenda. Motile organisms typically express active temperature preference or avoidance

responses that enhance or optimize their chances of survival and reproduction (Reynolds and Casterlin, 1979).

This study attempts to elucidate the relationship of temperature selection, thermal preference and activity in the crayfish <u>Pacifasticus</u> <u>leniusculus</u> artificially infected with killed <u>Aeromonas</u> <u>hydrophila</u>.

CHAPTER II

SURVEY OF LITERATURE

Background Studies in Febrile Response

In recent years various researchers have studied fevers
and febrile responses in an attempt to elucidate the
adaptiveness of this reponse and to determine the
mechanisms. Bennett and Nicastri (1960) raised questions as
to the function of fever and concluded that no strong
evidence was available to cite fever as beneficial or
harmful. High fevers or long term fevers have been noted to
cause brain damage, especially in neonates and in senescent
individuals (Kluger, 1976). The question of mild
temperatures as a physiological mechanism for combating
disease has only recently been re-evaluated as a positive
protective mechanism for inhibiting or alleviating certain
disease conditions, especially bacterial and viral
infections.

In endotherms, fever is considered a universal response
to endotoxins. Endotoxins as activators are components of
cell walls of gram-negative bacteria (Hokoma & Nakamura,
1982; Vaughn et al., 1974). Injection with live or killed
bacteria or with cell wall components (lipopolysaccharide
components-lipid A) leads to numerous physiological and

immunological responses including leukopenia, leucocytosis, mobilization of interferon, enhancement of nonspecific immunological resistance, reduction of serum iron and zinc titers, and fever production (Hacker et al., 1981; Hokama & Nakamura, 1982; Tocco et al., 1983). Viruses, hypersensitive reactions, tumors, and gram-positive bacteria have also been shown to elicite febrile responses in various animal species, even though gram-positive bacteria are not thought to produce endotoxins (Hokama & Nakamura, 1982; Kluger, 1979). Gram-negative and gram-positive bacteria both elicit a febrile response in various animal species; latent periods differ depending upon the animal species and the bacterial species. Pure endotoxin, now known to be the lipopolysaccharide coat of gram-negative bacteria, living or killed, produces fever within 15-30 minutes. Gram-positive bacteria require 45-60 minutes (Casterlin & Reynolds, 1977; Hutchinson, 1981; Monagas & Gatten, 1982; Kluger, 1976). Rabbits inoculated with virus demonstrate a febrile latency period of 1-2 hours post-inoculation (Wagner et al., 1949). Hypersensitivity reactions and tumors also elicit febrile response; the cause in the latter instance is still unknown.

Immunologic, phagocytic cells such as neutrophils, eosinophils, monocytes, liver Kupffer cells and macrophages produce endogenous pyrogen (Hokama & Nakamura, 1982; Kluger, 1979). The soluble agent originally called leukocytic pyrogen is now termed pyrogenic pyrogen because other cells

are now known to elicite febrile response as well (Kluger, 1979b).

Thermoregulation and Febrile Response

The concept of febrile response needs to be examined in light of thermoregulation and hypothalamic set points. Control of temperature is an extremely accurate system of sensing and responding to thermal fluctuations now considered to have evolved antecedent to development of the Classes Aves and Mammalia (Crawshaw et al., 1981; Hainsworth, 1981). The central nervous system in both endothermic and ectothermic vertebrates has been shown to be responsible for thermoregulation. Crawshaw and Hammel (1974) demonstrated the function of the anterior brainstem relative to thermoregulation in both bony and cartilagenous fishes.

The advantages for ectotherms of selecting particular ambient temperatures may vary; examples include reduction of metabolic need, enhancement of a physiological function, or selection of environs where vulnerability to predation is reduced. However, selection of elevated temperatures (those above normal preferred) is basically considered as a febrile response, especially when related to xenobiochemical agents such as viruses or bacteria.

The selected body temperature preference is now commonly stated in terms of set points or reference point temperatures in the hypothalamus. Alteration in the

hypothalamic temperature of ectothermic vertebrates will induce individuals to respond behaviorally as if the temperature of the entire body was altered. The brain, particularly the anterior hypothalamus and preoptic regions, is directly involved in fever production. During febrile states, the thermal set points or reference points appear to be raised. Crawshaw and Stitt (1975) showed that Prostaglandin E_1 (PGE_1), when injected into the preoptic/anterior hypothalamic region of squirrel monkeys, resulted in an increase in rectal temperature in a dose dependent manner. This response to prostaglandins occurs throughout the animal phylogenetic tree. Cabanac and Guelte (1979), utilizing scorpions <u>Buthus occitanus</u> and <u>Anductonus australis</u> from southern France and North Africa respectively, were able to demonstrate that these animals preferentially selected higher temperatures compared to the normal preferred temperature of $48.8 +/- 1.0^{\circ}$ C when injected with PGE_1. This febrile response demonstrated a dose dependent curve for duration and magnitude.

<u>Behavioral and Autonomic Fevers</u>

The mechanism for febrile response is controlled in endotherms by both autonomic and behavioral means (Crawshaw et al., 1981; Crawshaw & Stitt, 1975). Ectotherms exhibit only rudimentary autonomic responses which are of little thermoregulatory significance, but they can exhibit behavioral responses. Behavioral fever occurs when

individuals seek ambient temperatures which significantly increase their body temperatures above normally preferred levels. This response has been noted in turtles, fish, crayfish, scorpions, frogs, and lizards (Cabanac & LeGuelte, 1979; Casterlin & Reynolds, 1980; Hutchinson, 1981; Kluger, 1976; Monagas & Gatten, 1982; Vaughn et al., 1974). Scorpions (Class Arachnida) represent a phylogenetically ancient group of arthropods. If the fever response and thermoregulatory mechanisms occur in scorpions, then they are mechanisms which occur among the oldest known terrestrial animals (Cabanac & LeGuelte, 1979).

The significance of fever in response to bacterial infections was investigated first by Vaughn, Bernheim and Kluger (1974) using the lizard Dipsosaurus dorsalis as a model. In this study, elevation of body temperature affected the host resistance and consequently the outcome of survival. Survival of lizards infected with A. hydrophila increased from 0% at 34° C to 100% at 42° C. At 42° C, no deaths were attributable to bacterial infection. The relation between lizard body temperature (T^b) and percentage survival is highly significant. Increased survival rates of lizards at 42° C are attributed to enhancement of the host defense mechanisms, the result of temperature dependent mobilization of components of the immune response including leukocytic and phagocytic activity and humoral mediators. The 42° C survival success may

reflect both decreased bacterial growth and enhanced immune response. Control animals held at 42° C demonstrated significantly higher mortality, 34% compared to 0% in lower temperature (40°) controls. Sustained elevation of temperature (for <u>D. dorsalis</u> over 3.5 days) is lethal or at least potentially dangerous (Vaughn et al., 1974).

Most research indicates that pyrogenic responses, autonomic and behavioral, are fairly ubiquitous in metazoans as a defense against infections (Reynolds and Casterlin, 1976). However, the lizard <u>Cordylus cataphractus</u> (Cordylidae) does not show either response (Laburn et al., 1981) following injection with leucocyte pyrogen (LP) or attenuated <u>Aeromonas hydrophila</u>. Accordingly, Laburn et al.(1981) disagree with Kluger's conclusion that fever production is a phylogenetically ubiquitious, positive defense against infection.

Mediators of Febrile Response

As noted previously, several mechanisms or biochemical agents induce fever. Gram-negative and gram-positive bacteria, viruses, tumors as well as PGE_1 are involved. The mechanisms by which they induce fever is unclear.

Milton and Wendlant (1971) proposed that pyrogens might induce fever via the products of prostaglandins. PGEs are found in increasing amounts in the cerebrospinal fluid during febrile episodes induced by bacterial pyrogens (Coceani et al., 1983; Crawshaw and Stitt, 1975).

Researchers have proposed that PGE is formed in the central nervous system in response to pyrogens and acts at appropriate points in the thermoregulating pathways to cause an upward shift in the thermoregulatory set points.

Casterlin and Reynolds (1977) demonstrated that crayfish (Cambarus bartoni) injected with A. hydrophila and place in an aqueous environment with paracetamol added, produced normothermic temperatures, that is, no febrile response. Paracetamol is an antipyretic in vertebrates and is effective with PGE_1 and bacterial endotoxin. Previously, Casterlin and Reynolds (1973) demonstrated a PGE_1-induced fever in the same crayfish species.

Thermoregulation and Behavior

The major means of thermoregulation in ectotherms is behavioral, involving exploitation of the thermal heterogeneity of the environment. Some species are able to heat or cool their bodies physiologically . Aquatic species, especially the invertebrates and most vertebrate ectotherms, are strict poikilotherms and must thermoregulate behaviorally. Two necessary conditions necessary for behavioral thermoregulation are mobility and availability of temperature variations within the environment. These parameters need to be addressed in assessments of thermal preferendum and behavioral fever.

Animals exhibit intraspecific and interspecific ranges of thermoregulatory modes which vary with life cycle stages

or circadian cycles. For most aquatic invertebrates, parameters such as conduction, convection, radiation, and evaporation (limited to semi-aquatic individuals) are the means of temperature regulation. Aquatic ectotherms are, therefore, primarily poikilothermic, implying absence of regulation (Vernberg & Vernberg, 1979).

Most aquatic environments tend to exhibit little variation in microhabitat temperature except in those habitats exposed to stratification and/or solar radiance into a microhabitat (e.g., tide pools, shallow lake edges, exposed stream margins, or stratified standing ponds or lakes). Seasonal and circadian variation are also more common in these same microhabitats (Hynes, 1970; Vernberg & Vernberg, 1979; Mason, 1963; Taylor, 1984).

Aquatic invertebrates cope with varying temperatures in a variety of ways. Physiological adaptations include regulation of respiratory rates, changes in ventilation, and shifts in substrate pathways or enzyme concentration. Exposure to low temperatures, for example, increases oxygen consumption by the crayfish Orconectes virilis in correlation with shifts in Embden-Meyerhof and the hexose monophosphate pathways (McWhinnie & O'Connor, 1967). Integumentary pigment changes alter solar thermal radiation absorbance in fiddler crabs (genus Uca, Wilkens and Fingerman, 1965).

Evaporative cooling has been demonstrated in crabs
(Uca sp.and Ocypide macrocera, Vernberg and Vernberg, 1973),
isopods (Ligea oceanica, Edney, 1953) and in barnacles
(Tetraclita squamosa), limpets (Fissurella barbadensis), and
gastropods (Nerita tesselata) (Lewis, 1963). This status is
dependent on air exposure and changes with relative
humidity. Gunn (1942) stated that evaporative cooling is
not effective with truly aquatic organisms, and aquatic
invertebrates therefore, cannot have a body temperature
lower than the water.

Behavioral thermoregulation is possible in aquatic
species as in terrestrial ectotherms when thermal
variations in microhabitat are available. Crabs (Uca
pugnax) exhibit daily rhythms whereby individuals "bask" in
light during morning but avoid light during the hot midday
(Palmer,1962). Some species are able to effect evaporative
cooling (Edney, 1962; Wilkins & Fingerman, 1965).

Completely aquatic species are severely limited in
temperature regulation compared to terrestrial groups and
are likely to be strict poikilotherms. Terrestrial
ectotherms, both invertebrate and vertebrate, can regulate
more effectively than their aquatic relatives and therefore
in many situations are not strict poikilotherms. They often
exhibit homeothermic tendencies, taking advantage of various
microenvironmental differences to effect behavioral
thermoregulation in addition to physiological regulation.

Terrestrial organisms are able to orient toward or away from heat or cold, an opportunity presented by the generous array of terrestrial thermal microhabitats. Behavioral reactions resulting in animals favoring certain microclimates have an extremely important thermoregulatory function (Cloudsley-Thompson, 1970). Locusts (Schistocerca gregaria), for example, aggregate for warmth during cool weather and disperse during hot periods (Heinrich, 1970). Honey bees (Apis mellifera) and social wasps effectively control the temperature within the hive by both evaporative cooling and fanning during warm weather (Gary, 1975; Heinrich, 1970).

The South African scorpion Opisthophthalmas latimanus will move to its burrow entrance during the day and assume a stilting posture in order to cool itself. These species rely upon solar radiation or nocturnal reradiation for thermoregulatory control. Absorption of radiant heat is a common means of raising body temperatures and usually depends upon animal-controlled orientation to the sun's rays (Cloudsley-Thompson, 1970).

Insulating body structures (e.g., downey pubescence on honey bees Apis mellifera and sphinx moths Celeria linecta) and insulation by particular microhabitats (e.g., leaf packs, webs or subterranean hives) permit some terrestrial species to elevate body temperature (T^b) as much as 20° C above ambient temperature (T^a). These organisms, using

metabolic or physiologic methods, tend toward heterothermy similar to that seen in small mammals and birds (Allee et al., 1949; Adams & Heath, 1964). This thermogenesis derives from rapid vibratory contraction of thoracic wing muscles independent of wing movement.

Most aquatic species are unable to generate and maintain any appreciable quantity of physiological heat energy due to the high specific heat capacity and conductivity of water, the high surface to volume ratio of these small organisms, the poor insulating properties of their body structures, and/or their microenvironments. Some larger vertebrate ectotherms, such as sharks, tunas and crocodilians, are able to generate or conserve heat by various means including smaller surface to volume ratios and circulatory shunting of heat (Dizon, 1970; Prosser, 1973; Smith, 1970).

An adaptive response consisting of movement in a thermal gradient toward some selected temperature or preferred temperature is simple kinesis. This response has been noted in paramecia (Fraenkel & Gunn, 1969), snails, nematodes, and insects (Prosser, 1973). Fraenkel and Gunn (1961) coined the term klinokinesis, an extension and refinement of thermal kinesis, to refer to the reflex of turning away from a source of heat. A similar response to cold does not always occur, so animals may be trapped and/or immobilized. "A klinokinetic orientation is one in which

the frequency of turning or rate of change of the direction of movement is dependent on the intensity of stimulation" (Fraenkel & Gunn, 1961). For terrestrial but not aquatic species, relative humidity is an important parameter in terms of assessing preference and avoidance response but it does not affect aquatic species (Cloudsley-Thompson, 1970).

Activity behavior in O. obscurus increases in unfavorable temperatures and decreases in thermoneutral environments, thereby yielding a range of preferred temperatures (Crawshaw, 1974). Kerkut and Taylor (1958) and Prosser (1936) isolated heat sensative neurons in the abdominal ganglion of crayfish. The firing rate of these neurons was inhibited by increasing temperatures of the surrounding water and enhanced by decreasing temperatures. Cold-sensitive neurons have not been demonstrated (Pastro, 1981).

Crayfish (Orconectes immunis) demonstrate avoidance of temperature extremes in a gradient but do not prefer a particular temperature. Members of this species, like many other crayfish, are nocturnal (Crawshaw, 1974). Nocturnal mean temperatures, selected in a gradient from a broad range, averaged 22° C +/- 0.5° (Crawshaw, 1974).

Estimates of final preferendunm temperature for crayfish O. obscurus range from 29.8 to 33.9° C depending upon the method of calculation (Mathur et al., 1982).

Descriptive Parameters and Statistics

Because of broad variances in both range and mean values, both of which vary with individual animals and the experimental design, final preferendum for a species is probably best described as a range rather than a fixed temperature (Coutant, 1970). Factors contributing to different preferences may be individual variability and different behavioral activities, responses and needs, and physiological status (Brattstrom, 1970; Coutant, 1970; Laudien, 1973). Included with these effects are predator-prey interactions, various biorhythms or cycles, seasonal variations in nutrients, water depth or temperatures, and previous experience.

Temperature selection data typically are negatively skewed in distribution (DeWitt and Friedman, 1981). This fact is important for three reasons:

1. Upper lethal limits for most species are more sharply defined than are lower limits.

2. Animals respond more slowly to lower temperatures in their range, often becoming trapped or immobilized by low temperatures (Cloudley-Thompson, 1970).

3. Optimum physiological rate process, which is most closely associated with preferred temperature (Tp), tends to exist toward the upper end of an animal's range of tolerance.

Dewitt and Friedman (1979) hypothesized that negatively skewed distributions are based on physiological rate process, an exponential function of temperature. This idea is consistent with the function described by parameters such as Q_{10} and u.[1] Many tissue and cellular functions proceed optimally near preferred body temperature -capacity adaptations (Huey and Stevenson, 1981; Laudien, 1973).

Loring and Hill (1976) found that the crayfish $\underline{O}$. causyei showed high variability during acute preference tests, especially during initial test periods. Biased values resulting from inactivity of particular individuals occurred at low temperatures. Skewed data may result from quiescent or hyperkinetic activity in some individuals exposed to artificial laboratory designs.

Proper characterization of the thermal sensitivity of various physiological systems requires at least three descriptive parameters: optimal temperature, thermal performance breadth, and tolerance range- expressed in terms of lethal limits (Huey and Stevenson, 1981). Optimal temperatures and thermal breadth account for values whereby capacity adaptation is most successfully achieved. Tolerance range implies those values which define any activity or survival and thus reflect resistance adaptation (Precht et al., 1973; Huey and Stevenson, 1981; Laudien, 1973).

[1] Q_{10} and u are designated as the temperature coefficient and the energy of activation, repectively.

CHAPTER III

MATERIALS AND METHODS

Collection and Housing

A total of approximately one hundred crayfish
(<u>Pacifasticus</u> <u>leniusculus</u> <u>trowbridgii</u>, Miller,1965) were
collected on two separate occasions from the Clackamas River
five miles east of Gladstone, Clackamas County, Oregon. The
animals ranged in weight from 3.9 to 29.6 g and in length
from 48 to 90 mm, measured from the end of the telson to the
tip of the rostrum. Animals were housed in an environmental
chamber at a constant temperature of 10° C and a constant
light : dark cycle ratio of 13 : 11 hours. These conditions
approximated the environment at the times of collection time
and were maintained in order to reduce captivity stress and
induction of molting which may affect an animal's hormonal
status and ultimately one's experimental results.

Within the chamber, six to ten animals, depending on
their size, were maintained in aquaria with subgravel
filters and a one inch gravel layer. Protection and burrow
sites were provided in the form of broken claypots, slate
slabs, or PVC pipe sections. Aeration was enhanced by the
action of the filter air bubbler unit. The temperature of
10° C, in addition to mimicking environmental conditions at

collection, allowed for monitoring movement of animals toward a higher thermal preferendum during the experiments. Lighting was supplied by regular and full spectrum Grow-Lux fluorescent fixtures. Animals were fed weekly with pieces of salmon and vegetable matter.

Crayfish used in the gradient were randomly selected for size, sex, and location in the environmental chamber. Individuals in treatment groups were numbered and color-coded on the carapace prior to an experimental run. After experimental trials, animals were returned to a specified treatment aquarium in order to monitor health and status of individuals in specific treatment groups.

Gradient Design and Data Collection

All treatment groups were assessed in an aquatic thermal gradient (Crawshaw, 1974). The schematic diagram of the gradient apparatus is depicted in Figure 1. Each of the ten chambers in the gradient measured 20 cm wide by 22.75 cm long.

The apertures between the baffling partitions were 15.5 cm wide. The approximate water depth of the gradient was 10 cm, maintained over a coarse gravel bed of 2 cm to provide traction and a degree of cover for the crayfish, which tend to burrow during the light periods if other cover is unavailable. The total water volume for the gradient filled to a depth of 10 cm was 44 1.

Light cycles for the gradient were maintained close to the conditions of the environmental chamber. Animal activity, temperature selection, and location in the gradient were typically recorded for 10-hr periods.
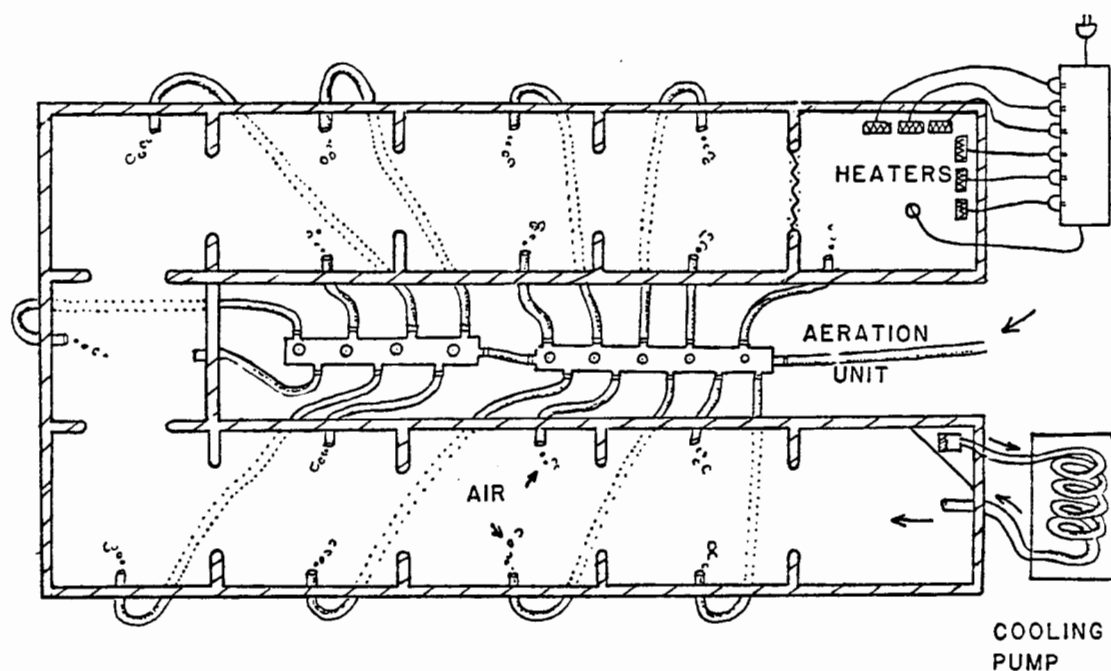
Figure 1. Schematic diagram of gradient apparatus.

Movement of crayfish relative to temperature selection (position in the gradient) was recorded with a 36 gauge copper-constantan thermocouple secured to the carapace of the animal prior to placement in the gradient. The thermocouple was glued to experimental animals at the dorsal fusion line of the cephalothorax using waterproof rubber

cement and a string loop. The string was looped between the coxopodites of the chelipeds and first walking legs, girdling the brachiostegite. The thermocouple wire was suspended from a central point one meter above the gradient in order to facilitate free movement of the crayfish through the gradient.

Millivolt readings from the thermocouple were amplified and transcribed by a Fluke #8810A digital meter to a Vic 20 Commodore computer programmed to convert millivolt readings of the Fluke to degrees Celsius. Readings taken every ten seconds were averaged over a five minute interval; these means were used to compute a median preference temperature.

Inoculant Preparation

Aeromonas hydrophila, a facultative gram negative bacillus known to be a ubiquitous waterborne pathogen, produces red leg disease in anurans and septicemia and stomatitis in reptiles (Bergy's Manual, 1974). The bacteria, obtained from Carolina Biological Supply, were cultured by inoculation into 50 ml of nutrient broth. The medium was incubated at 37° C in a 125 ml Klett flask and aerated with agitation at 150 rpm in a NBS gyrotory shaker for 8 hr.

Growth suspensions were monitored at 600 nm on a Klett-Summerson photocolorimeter (model 800-5). Viable colony plate counts were used to enumerate the bacteria in suspension for readings of Klett 100. Counts were completed on serial dilutions of 10^{-6} and 10^{-7}.

Actual inoculation doses of killed bacteria were prepared by centrifuging 20 ml of suspension for 5 min at 48 G. The pellet was resuspended in 20 ml of 70% ethanol for 10 min. The effectiveness of the ethanol was confirmed by plating 0.1 ml of killed bacterial suspension on nutrient agar and culturing. The killed bacteria were recentrifuged and the pellet washed with 0.9% pyrogen-free NaCl solution. The wash procedure was performed three times. The final pellet was resuspended in 5 ml of 0.9% pyrogen-free saline. Final doses of 6×10^7 were made by dilution with additional saline. Sterile pyrogen-free rinses and solutions were obtained using deionized double-distilled water. Inoculation stock was periodically tested for contamination during the experiment period by soft agar plate count (Atlas et al., 1984).

Experimental Treatment Groups

The experimental design consisted of three treatment groups each with nocturnal and diurnal runs in the gradient. The baseline treatment (uninjected/uninfected crayfish) and infected treatment (n = 10) were paired, the same animals were used in both treatments. Saline control animals (n = 7) were independent.

The infected treatment group was established by injecting approximately 6×10^7 bacteria suspended in 0.2 ml of 0.9% pyrogen-free saline into the proximal portion of the coxopodite of the cheliped. This dose was initially based

upon concentrations cited by Casterlin and Reynolds (1979) in work with Cambarus bartoni. Doses ten fold larger were fatal for P. leniusculus in the majority of cases tested, or left specimens comatose. Doses ten-fold less produced no discernable response, either behaviorally in terms of posture and demeanor or in terms of behavior in the gradient relative to temperature selection.

Each animal was placed immediately in the cold end of the gradient at approximately $8-10^{\circ}$ C, corresponding with the holding temperature of the environmental chamber. Experimental runs of each group were consecutive within the group; therefore, all baseline animals were run prior to control and experimental runs. After baseline runs, animals were returned to the environmental chamber and housed as a group. Other than the injection, all treatment groups were handled identically.

Microscopic inspection of control samples specimens revealed that the bacteria were dispersed throughout the coelomic cavity shortly after the injections were made.

All runs (each treatment) began at approximately 2000 hr (night cycle). Day treatment temperture recording (for the same animal) began at 0800 hr, following termination of night treatment recordings. Data utilized for analysis were from the first eight hours of both night and day treatments.

Data and Test Parameters

Data for individual animal responses were evaluated in terms of median values, interquartiles, and 95% minimum/maximum range, as opposed to mean and standard deviation. Median, as a measure of central tendency, coupled with range and/or interquartile values can be a better estimate of central tendency than mean for several reasons (DeWitt and Friedman, 1981; Huey and Stevenson, 1981; McCauley, 1977; Sokal and Rohlf, 1969).

Medians are less influenced than arithmetic means by outlier values. Negatively skewed distributions, which seems to be an inherent characteristic of this type of data, has less effect on the median as a central tendency, whereas, the arithmetic mean is sensitive to asymmetric distributions (Sokal and Rohlf, 1969). Physiological processes exhibit optimum ranges and central values, but may be characteristically influenced by an animal's environmental or physiological status.

Individual median values were used to compute the mean and standard error of a particular treatment population. These medians, as measures of individual central tendency, were thus used as data points to establish a central tendency for each treatment group. Treatment groups were compared in terms of mean values.

Treatment groups were also compared according to levels of activity. Activity of treatment animals was

calculated as the ratio of changes in temperature selection
>1.5^o C per hour. The unit of 1.5^o was selected as the
average temperature change between gradient compartments,
computed as gradient temperature range difference (15° C) /
number of compartments (10). A change >1.5^o C was recorded
as one activity unit and considered as an active locus
change by the crayfish. The 1.5^o C value offsets local
temperature fluctuations within the gradient and reflects a
significant movement from one locus to another.

Active changes in direction (klinokinesis) of specimens
in the gradient were determined by tabulating rising or
falling temperature changes (temperature inflection) >1.5^o
per hour. Directional changes are supplemental to measures
of activity stated previously.

Range values- the difference between the 95%
minimum/maximum values- were used to compare variability in
overall movement throughout the gradient.

Statistical Tests and Results

One-way ANOVA and Student-Newman-Keuls tests (Sokal and
Rohlf, 1969; Zar, 1984) were applied to data for diurnal and
nocturnal values of saline, baseline, and infected groups.
Mean (preferred) temperatures, activity units, temperature
inflection values, and 95% minimum/maximum range
differences were analysed.

Student's t-test was also applied to the g_1 statistic,
an indicator of normality or skew in a frequency
distribution (Sokol and Rohlf, 1969).

CHAPTER IV

RESULTS

Tables and Figures for Data

Table I lists median preference temperatures, first and
third quartiles, and minimum/maximum 95% values (range) for
individual animals in each treatment group. These data are
shown in Figures 2 and 3 with notched box plots (Chambers et
al., 1983). Ranges (minimum/maximum 95%) are indicated by
the vertical lines (whiskers), median values (notch line)
are the horizontal lines, and semi-quartile values are the
box ends. The mean value of each box plot graph is
represented by the horizontal line through the center of
each graph. Figure 2 compares individual animals for
nocturnal treatments, within and across treatment groups;
Figure 3 compares diurnal data.

The box plots provide graphic demonstration of the
similarities and differences occuring within and between the
treatment groups. In Figure 2, the box areas (1st-3rd
interquartile) for the infected animals are discernably
narrower than the boxes representative of the baseline and
saline animals. In contrast, comparisons of the same
parameters for Figure 3. indicate that the boxes are similar
easily identified- for nocturnal treatment animals, RS12 and
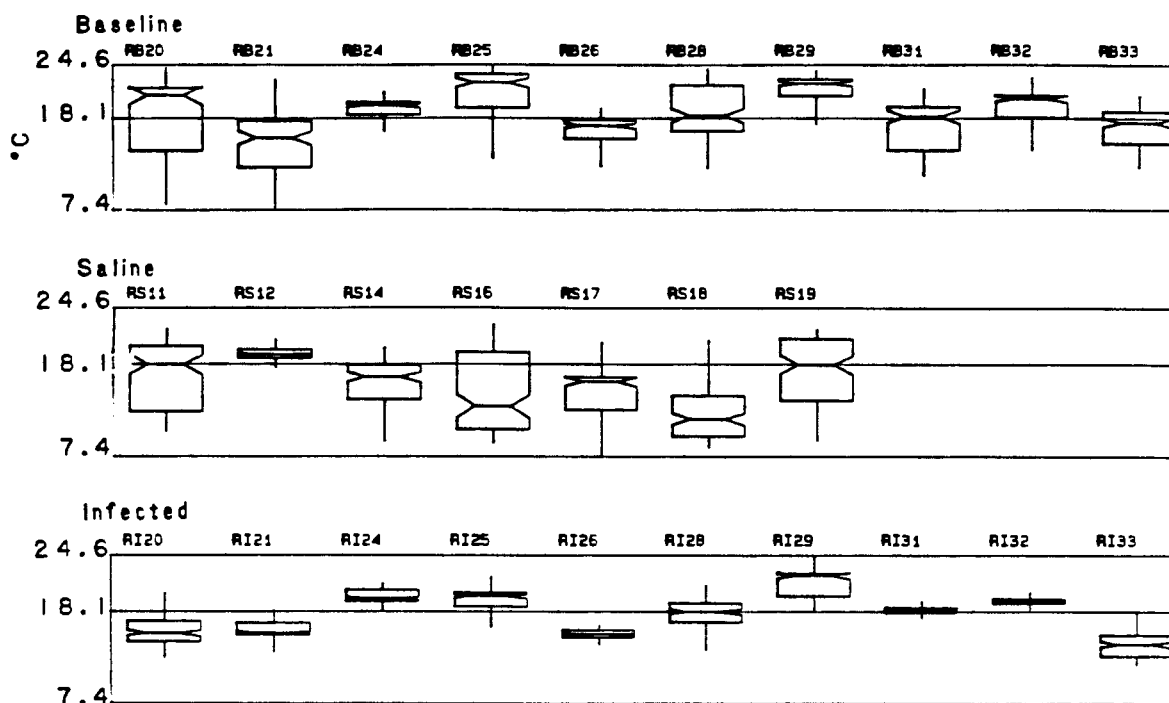RB24 were basically inactive in the gradient; for diurnal

TABLE I

MEDIAN AND RANGE DATA FOR INDIVIDUAL SPECIMENS PER TREATMENT GROUP

SALINE TREATMENT

Specimen Id.		RS11	RS12	RS14	RS16	RS17	RS18	RS19
Max. 95%	D	20.70	19.90	18.60	21.00	19.90	15.00	22.90
	N	22.20	21.00	20.00	22.70	20.60	20.90	22.10
3rd Quart.	D	18.90	18.80	17.40	19.60	18.80	14.70	22.10
	N	20.20	19.80	18.00	19.50	16.60	14.45	21.10
Median	D	18.65	18.20	17.10	19.30	11.25	14.50	21.70
	N	18.05	19.20	16.60	13.20	16.10	11.75	18.05
1st Quart.	D	16.50	17.90	16.35	18.35	10.90	14.40	20.80
	N	12.60	18.80	14.00	10.55	12.80	9.80	13.90
Min. 95%	D	15.80	16.70	15.10	17.00	10.00	14.00	19.00
	N	10.30	17.70	9.20	9.00	7.70	8.50	9.30

BASELINE TREATMENT

Specimen Id.		R20	R21	R24	R25	R26	R28	R29	R31	R32	R33
Max. 95%	D	26.70	11.30	20.40	26.00	19.00	19.80	22.30	22.70	22.30	20.00
	N	24.20	22.80	21.20	24.40	19.20	24.10	23.90	21.60	23.10	20.70
3rd Quart.	D	24.50	10.80	19.60	24.20	18.30	19.50	21.40	20.70	20.60	19.50
	N	21.70	17.80	20.00	23.50	17.90	22.10	22.90	19.50	20.80	18.90
Median	D	23.80	10.20	19.20	23.80	17.90	19.40	20.80	19.40	20.30	10.80
	N	20.80	15.80	19.60	22.40	15.60	18.40	22.30	18.30	20.40	17.60
1st Quart.	D	23.00	9.60	18.20	22.30	17.20	19.30	20.50	18.30	19.00	18.70
	N	14.30	12.40	18.50	19.30	14.70	16.60	20.70	14.40	18.20	15.20
Min. 95%	D	21.20	9.00	16.20	19.50	15.70	19.00	19.70	15.10	16.70	18.40
	N	8.10	7.40	16.60	13.40	12.50	12.30	17.50	11.50	14.40	12.40

INFECTION TREATMENT

		R20	R21	R24	R25	R26	R28	R29	R31	R32	R33
Max. 95%	D	21.40	17.60	19.60	22.40	19.70	17.40	18.20	19.00	19.30	13.90
	N	20.20	18.20	21.30	22.10	16.50	21.10	24.60	19.30	20.30	17.90
3rd Quart.	D	20.80	17.20	19.50	21.30	19.00	17.30	16.90	18.80	18.40	13.80
	N	17.00	16.80	20.60	20.30	16.00	19.10	22.50	18.60	19.60	15.40
Median	D	20.50	17.10	19.30	21.10	18.80	17.20	16.60	18.70	18.10	13.70
	N	15.60	15.60	19.60	19.90	15.50	18.10	22.30	18.30	19.30	14.30
1st Quart.	D	18.60	16.50	19.00	20.10	18.50	17.20	15.50	18.60	17.80	13.70
	N	14.60	15.40	19.30	18.70	15.10	16.90	19.90	18.00	19.10	12.90
Min 95%	D	17.90	15.90	18.60	19.00	18.10	17.10	14.10	18.30	17.00	13.60
	N	12.80	13.40	18.30	16.30	14.30	13.70	18.40	17.40	18.40	12.00

[1] D/N represent diurnal and nocturnal values

Figure 2. Notched box plots of nocturnal treatments
comparing medians (box notches), 1st/3rd interquartiles
(box ends), & 95% min/max ranges (vertical whisker
lines) for individual animals. Mean group values are
the central horizontal lines running through the graphs

ACROSS GROUPS

Baseline

Saline

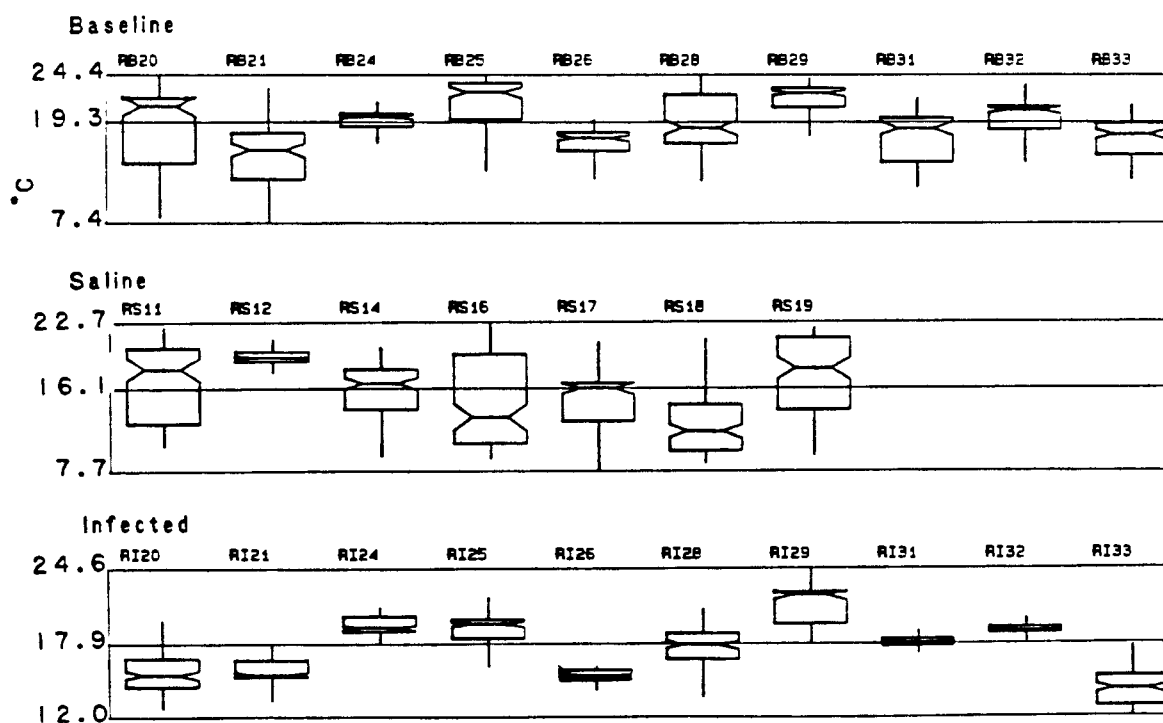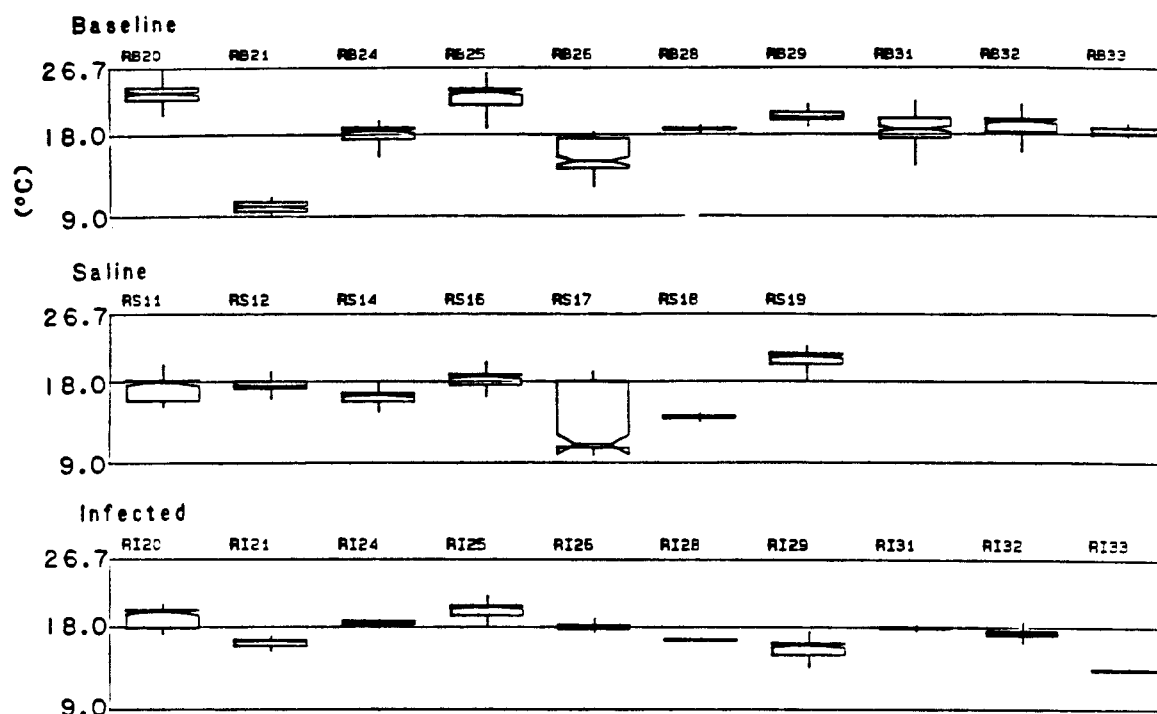Infected

WITHIN GROUPS
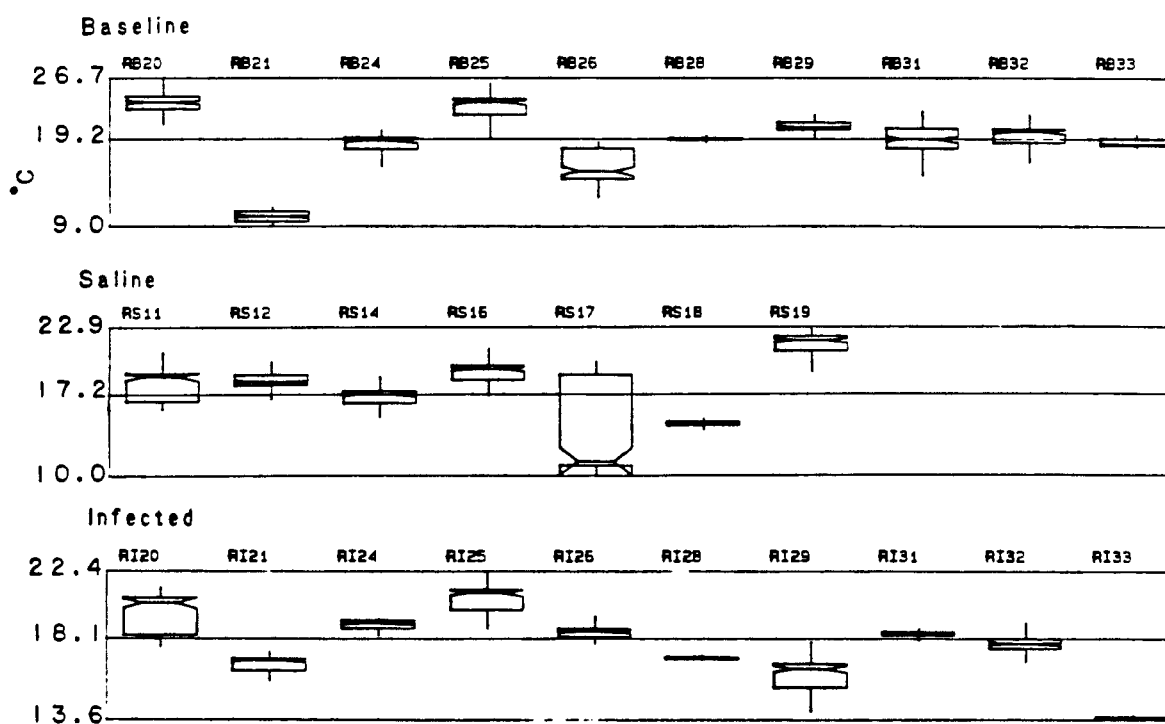
Baseline

Saline

Infected

Figure 3. Notched box plots of diurnal treatments
comparing medians (box notches), 1st/3rd interquartiles
(box ends), & 95% min/max ranges (vertical whisker
lines) for individual animals. Mean group values are
the central horizontal lines running through the graphs.

treatments, animals labelled RB26, RI20, and RS17 show more activity than other animals in their respective groups.

Large areas below the box notch (median line) are indicative of negative skewing. For inactivity, whether the result of light period or infection, the reduction of negative skew accompany the overall reduction in activity and range. This fact is demonstrated by the narrow interquartile areas and the more nearly equal upper and lower regions of the boxes. These results concur with the presentation of normal probability percentages (used to depict skew) of pooled data graphed in Figure 6 (see description below).

Table II contrasts mean values and standard errors for preferred temperature, 95% minimum/maximum range, range difference, activity, and direction change for each treatment group.

Figure 4 shows temperature selection (ordinate) versus time (abscissa) for representative animals from infected and baseline (uninfected) treatment groups. Each data point is a 5-min mean interval reading. Diurnal and nocturnal data are contrasted in each graph. Graph values originate at $8-10^{\circ}$ C, the beginning temperature for each run and the acclimation temperature of experimental animals. The klinokinetic response is most obvious in the nocturnal plot for baseline. At night, the uninfected animal typically moves from one temperature extreme to the other within a

selected temperature range. This response is considerably reduced in the diurnal plots of both baseline and infected treatments as well as in the nocturnal plot for the infected animals.

TABLE II

SUMMARY Of MEAN AND STANDARD ERROR VALUES FOR
PREFERRED TEMPERATURE, 95% MIN/MAX RANGE,
RANGE DIFFERENCE, ACTIVITY,
AND DIRECTION CHANGE

TREATMENT GROUP	PREFERRED TEMPERATURE ^{o}C [d]95% MIN/MAX RANGE ^{o}C	[a]ACTIVITY RATE	[b]DIRECTION REVERSAL RATE	[c]RANGE DIFFERENCE ^{o}C
Baseline (n=10)				
Diurnal	19.2 +1.25 16.7 +3.72/21.1 +4.27	*0.20 +0.04	*.12 +0.02	*4.32 +0.74
Nocturnal	19.3 +0.69 12.6 +3.21/22.5 +1.77	0.39 +0.03	0.23 +0.02	9.91 +1.19
Saline (n=7)				
Diurnal	17.2 +1.29 15.4 +2.84/19.7 +2.46	*0.14 +0.03	*0.10 +0.20	*4.34 +1.03
Nocturnal	16.1 +2.73 10.2 +3.38/21.4 +0.99	0.35 +0.05	0.20 +0.02	11.11 +1.34
Infected (n=10)				
Diurnal	18.1 +0.67 17.0 +1.87/18.9 +2.34	0.80 +0.02	0.06 +0.01	1.89 +0.43
Nocturnal	17.9 +0.08 15.5 +2.52/20.2 +2.33	#0.19 +0.03	#0.11 +0.02	#4.65 +0.70

[a] computed as changes >1.5 ^{o}C per hour
[b] computed as rising or falling changes in temperature > 1.5 ^{o}C per hour
[c] difference between 95% minimum/maximum values in ^{o}C
[d] first value is minimum 95%, second value is maximum 95%, or lower and upper range values
* indicates significant difference within groups, diurnal and nocturnal values
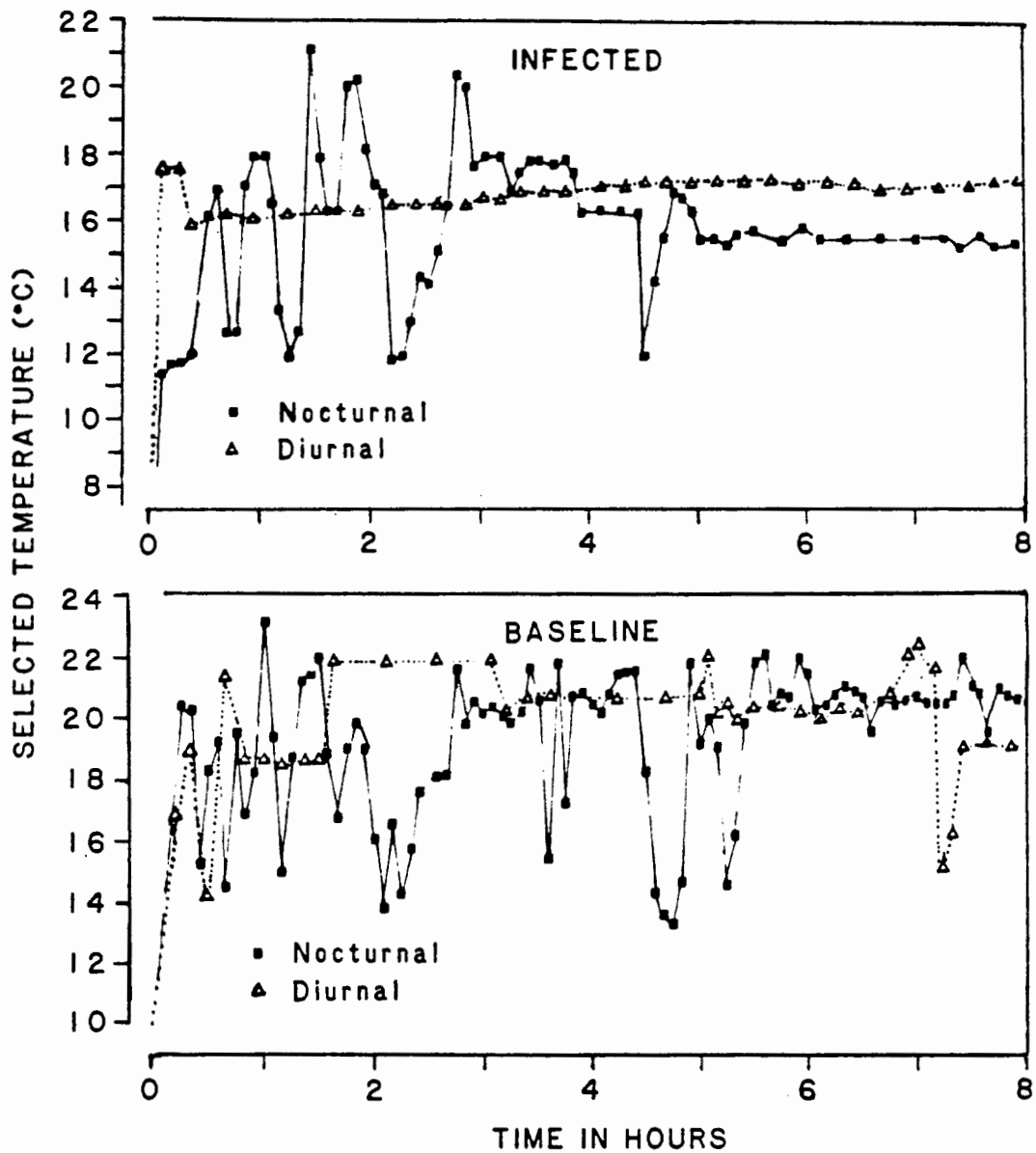# indicates significant difference across groups, nocturnal only

Figure 4. Representative examples of baseline and infected animal: selection of temperature over time.

The activity of the infected animal diminishes at about three hours, indicating when the bacterial injection is causing an observable effect. The lower temperature range value does not include the acclimation temperature in this example; this is also true for most of the animals tested.

Figure 5 demonstrates cumulative frequency percentages for pooled values of each nocturnal treatment group, plotted as temperature selection (abscissa) versus cumulative frequency (ordinate). The slope of the major portion of the curves for the day treatment data and for the night treatment for infected animals is steeper than for baseline and saline night treatment data. This indicates a narrow preferred range in these treatments and correlates with reduced activity.

The data for nocturnal treatments from Figure 5 are replotted in Figure 6 using a normal probability scale in order to depict the nature of the distribution (normal or skewed). The frequency distribution of both the baseline and saline treatment (nocturnal) are negatively skewed, as depicted by an upward curve (as the lower end of a sigmoid curve); straight lines are indicative of normal distributions (Zar, 1984). The infected group curve is typical of a leptokurtic distribution –a narrow grouping with most values falling very close to the mean or central
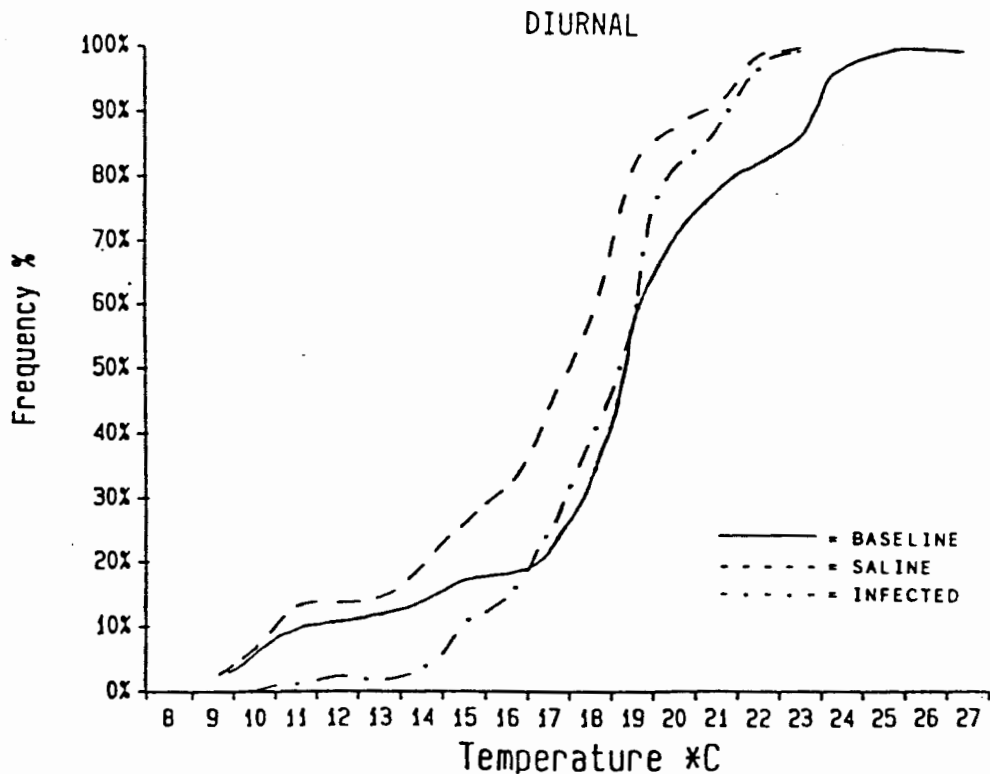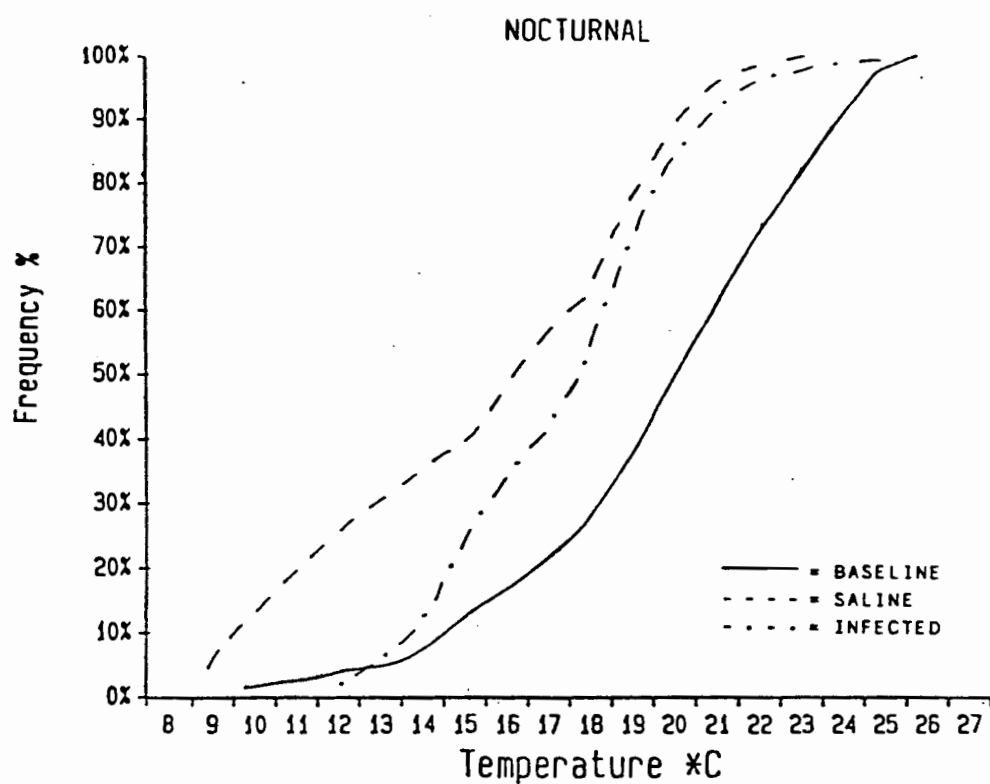
Figure 5. Cumulative frequency percentages of pooled temperature selection data from each treatment group.
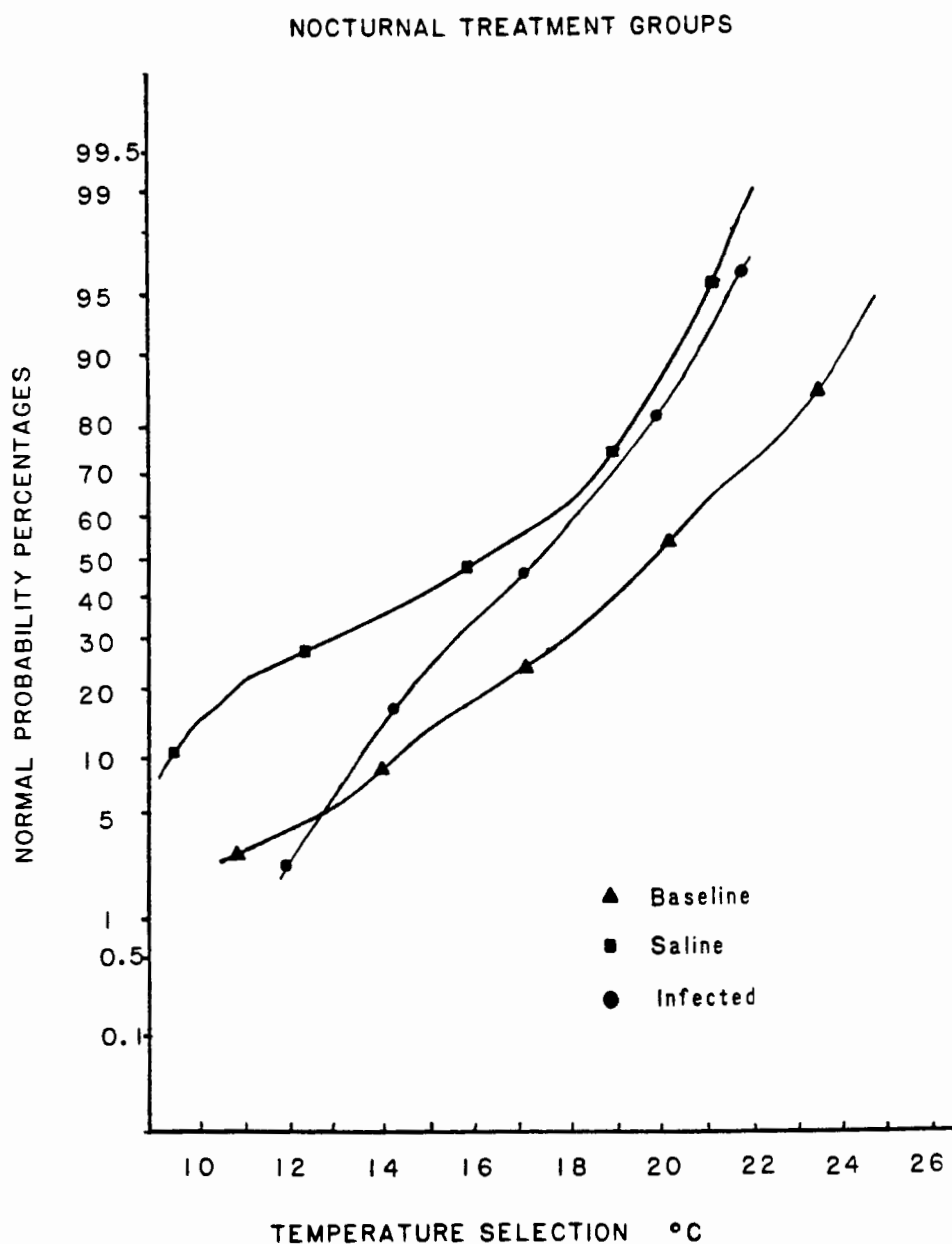
NOCTURNAL TREATMENT GROUPS

Figure 6. Cumulative frequency percentage data from Figure 5 replotted on normal probability paper. Baseline and saline plots indicate significant negative skew in the temperature distribution curves. Infected treatment plot is indicative of leptokurtic distribution with some negative skew tendencies.

value and trailing in the tail regions of the distribution
(Sokal and Rohlf, 1969).

Mean temperatures among the six treatment groups were
not significantly different (F= 1.38, with 5 and 48 degrees
of freedom; P> 0.05). Activity, directional movement, and
range were significantly different (F=15.4, F= 14.18 and
F=15.15, respectively, with 5 and 48 degrees of freedom; P>
0.05).

Values for activity, number of directional changes, and
temperature range difference, for infected animals in the
nocturnal treatment were significantly different from values
for baseline and saline treated animals. Infected, saline,
and baseline values from diurnal tests were significantly
different from saline and baseline values from nocturnal
tests, but not significantly different from each other or
from values from infected animal tests. Restated, night
infected data are not significantly different from diurnal
data but are significantly different from other nocturnal
data.

A t- test of the skewness statistic g for pooled
frequency data from each treatment group indicates that all
treatment temperature selection curves are significantly
negatively skewed (P> 0.001). Temperature distributions of
individual animals were negatively skewed in 87% of the
cases (all treatment groups included) based upon a g
statistic for each distribution. As previously stated,

graphic analysis of the distribution curves (Figure 6) indicates a negative distribution for the pooled date for the three nocturnal treatments. Diurnal treatment distribution curves (not shown) are similar to the distribution curve for temperatures selected by infected animals in the nocturnal treatment. In addition to being negatively skewed, diurnal and 'infected' night treatment distributions are leptokurtic.

Behavior of Infected Animals Compared to Controls

Prior to being infected, animals were aggressive, actively challenging any visual stimuli or movement with raised, open chelipeds. During handling, the animals were capable of powerful gripping ability with the chelipeds (measured by my inability to remove the crayfish from my hand once they clasped it). Uninfected animals stood with legs splayed out and body raised off the substrate. Uninfected and saline control animals displayed normal defensive behavior during all aspects of the experiment.

Infected animals, on the other hand, did not actively display a defensive posture. Strength in the cheliped grip was reduced to the point of being ineffective. The animals held the walking legs tucked inward and forward with the body appressed to the substrate.

Infected individuals (diurnal and nocturnal) and noninfected diurnal animals sequestered themselves in the corners formed by the baffle partitions in the gradient.

This behavior resembles hiding behavior of animals in the wild. Healthy animals also tended to partially bury their vulnerable abdomen into the gravel. Infected animals were much less likely to attempt to flee or defend themselves, whereas healthy animals did so vigorously.

All baseline and saline treated animals survived at least one week past the experimentation periods. Of the infected group, three deaths were directly attributable to the effect of the treatment. One death occurred at each of 48, 96, and 120 hr post inoculation. The remaining seven animals regained strength and stamina over the course of several weeks, and at least one molted. Data from animals which died during the twenty-four hour trial period were not utilized in the comparisons.

CHAPTER V

DISCUSSION

Comparison of this Study to Other Studies[

In this study, crayfish infected with _Aeromonas_
hydrophila did not select a thermal preferendum
significantly different from that selected by uninfected
crayfish. However, infected animals did select temperatures
in a very narrow and specific range, and they tended to
stay in one temperature locus once it had been selected.

The data from the temperature preferendum aspect of
this experiment are contrary to most of the results
published by other investigators. Only the Laburn et al.
(1981) study of cordylid lizards noted no significant change
in temperature preferendum for infected versus uninfected
animals.

Assessment Relative to Environmental Parameters

Pelagic aquatic animals such as temperate and tropical
fish and terrestrial animals have greater access to broader
fluctuations and variability in their environment than do
many benthic aquatic species (Hynes, 1970; Williams, 1981).
P. leniusculus inhabits fast-moving streams and rivers as
well as ponds and lakes. In moving water these crayfish
typically seek shelter beneath rocks and anchored wood

debris. Nocturnally, the crayfish forage on the rocks and other substrate; activity commences at dusk. Similar observations were noted for Cambarus immunis (Crawshaw, 1974). In fact, T.H. Huxley (1884) stated that this behavior of crayfish to be common knowledge.

Pacific coast rivers and streams, where P. leniusculus resides, are relatively cold, fast-moving waters fed by mountain snowmelt. These water courses are short in length, allowing little chance for the warming effect more common to eastern streams and rivers. Williams (1981) noted that fast-moving rivers and streams offer little variaton in temperature due to mixing and turbulence. Additionally, streams are typically shaded. In those regions where tree canopies are open as a result of farming or logging, warmer pools or shallows may occur. However, this factor would be, at best, present only if long intervals of insolation occurred. The effect is diminished during the typical nocturnal activity periods of the crayfish.

Other studies indicate that crayfish use various environmental cues (e.g. temperature or light) to select shelter or migrate to regions where risk of predation is reduced. One species, O. immunis, selects lower temperatures during the day (18° C) than at night (20° C) (Crawshaw, 1974). In contrast, P. leniusculus does not select significantly different temperatures.

As additional examples, ovigerous females (O. immunis) use temperature cues to move to deeper water in order to segregate from males (Stein, 1977). Crayfish O. propinquos modify their behavior and microdistribution relative to predation by largemouth bass (Micropterus dolomieui) Stein, 1977).

Seeking shelter in daylight reduces vulnerability. During collection of P. leniusculus for this study, only larger individuals were found exposed during the daylight hours and mostly in areas of prime forage (e.g., near salmon carcasses). Crayfish were typically collected from beneath rocks or logs. In small woodland streams animals segregate according to size; small individuals (< 55mm) select riffles and glides, whereas larger animals inhabit deep pools or hide beneath larger rocks or logs. This segregation reduces cannibalism (Mason, 1963).

Shelter affords the only protection for infected animals since flight or defense behaviors are ineffective against predators and cannibalism. During infections, if a temperature choice is possible, a conflict between the best option for survival may result. More than likely, shelter may be the primary need. For uninfected crayfish (O. causyei), shelter within the temperature range (resistance capacity) is dominant to preferred temperature (adaption capacity) (Loring and Hill, 1976). In this study of P. leniusculus, infected animals demonstrated a restricted

range for preferred temperature with little individual deviation from the mean. The significance of the narrow preferred temperature might be better elucidated if studied in conjunction with accessibility of shelter.

Infected animals in this study actively selected a preferred temperature. This fact is demonstrated by movement of infected individuals away from the acclimation temperature (initiation temperature in the gradient of $8-10^{o}C$) toward a group mean value of 17.8^{o} +/- .80 for diurnal tests and 18.1^{o} +/-.67 for nocturnal tests. During trial runs, infected animals which were moved to the cold or warm end of the gradient moved back to the preferendum region. This procedure was performed to determine if animals were able to distinguish temperature change and positively select a preferendum point. By contrast, in a thermally neutral "gradient", infected animals did not change position or select a different location.

Further analysis of the relationship between shelter, infection, and preferendum temperature should improve understanding of the significance of febrile temperature selection for some species. Additional significance might be established, for instance, by studying additional species which are not afforded access to extensive gradients in their environment, e.g., arctic fish or benthic marine species.

CHAPTER VI

CONCLUSIONS

1. The crayfish _Pacifasticus leniusculus_, when infected with the gram negative bacterium _Aeromonas hydrophila_, does not select a thermal preferendum significantly different from that preferendum selected when uninfected.

2. Infected animals restrict the variance and range of temperatures selected. This implies that this temperature is the appropriate value for these animals to maintain a physiological and immunolgical reponse.

3. In their natural habitat, these crayfish have access to a limited range of temperature during most of their annual (general) and nocturnal (specific) activity periods. Febrile response may be less important than maintaining an accurate and precise temperature level within the preferred range (not activity range). In order for a behavioral febrile response to function, a thermal choice is essential.

4. Reduction of activity is a behavior related to the stress of infection. When infected, these animals do not possess adequate defense capabilities nor competent flight skills. Infected behavior is similar in several respects to diurnal behavior, for example, a reduction in activity and

an increase in hiding. Diurnal behavior reduces risks due to predation. As in the study by Loring and Hill (1976), shelter or hiding for this species may be more significant than a specific temperature preference if there is a discrepancy between the two parameters.

   5. Animals acclimated to 10° C prefer a mean temperature higher than the acclimation temperature. The difference in these temperatures is about 6 C degrees.

   6. Uninfected animals, during a dark period, translocate between temperature extremes of a selected temperature range. Reversal of direction in the gradient is established primarily after encountering unacceptable high or low temperatures.

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