OSMOREGULATION IN TWO GEOGRAPHICALLY ISOLATED POPULATIONS OF FRESH WATER CRAYFISH

by

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INTRODUCTION

The class Crustacea can be considered mainly a marine group. There are several forms, however, which have invaded fresh water. One of the most successful of these is the fresh water crayfish. There are areas on the Pacific Coast where the crayfish Pacifastacus is found in brackish water, which might appear to be a reinvasion of the marine environment. It was felt that a comparison of the osmotic behavior of a brackish water form and fresh water form might help explain the presence of these animals in brackish water.

Physiological, anatomical and geological evidence indicate that the ancestral forms of the major phyla and most of the major classes originated in the sea. Since this origin, sea water has changed little in the way of offering the marine organism an ideal environment (2, p. 172).

As ancestral forms began to invade land, brackish water and fresh water, regulation of internal osmotic concentrations became inevitable. With respect to osmotic behavior, aquatic life can be found in five ecological environments: (1) marine, (2) brackish water, (3) fresh water, (4) semi-terrestrial, and (5) inland saline water.

These categories are not fixed entities, there being many intergrades between the groups. The first four are important in clarifying the existence of fresh water forms, while the fifth is only of special interest and will not concern us here.

Evidence indicates that ionic regulation was established early. This was probably due in part to the change in relative ionic concentration of the sea. With few exceptions, ionic regulation is universal among existing marine organisms (2, p. 173; 21, p. 10). Cole (6, p. 583), however, states that the Echinoderms are not among the ionic regulators. Robertson (27, p. 387-397; 28, p. 277-296) found that among the 34 marine invertebrates he studied, belonging to the Echinoderms, Annelida, Sipunculoidea, Mollusca, and Arthropoda; that only Echinus, an Echinoderm, was not an ionic regulator. Echinus was in complete ionic equilibrium with its environment.

It has not been shown that true marine animals which spend their life in the ocean have any need for regulating their internal osmotic concentration. The blood of these animals is in osmotic equilibrium with the sea in which they live. Their blood can be said to be isotonic. In the study mentioned above (27, p. 387-397; 28, p. 277-296) only Pachygrapsus was found not to be isotonic to its

environment. Pachygrapsus is a shore crab that has invaded a niche which necessitates some sort of osmoregulation.[1] When marine animals are subjected to various dilutions of sea water the osmotic concentration of blood usually follows that of the medium. The crabs Cancer antennarius, Lophopanopeus heathii and Speocarcinus californiensis behave in this fashion (14, p. 82). The common sand crab Emerita analoga (11, p. 47) and the spider crab Maja (21, p. 24) behave similarly.

Four species of the amphipod Gammarus, presumably representing an evolutionary sequence from marine to fresh water, were studied by Beadle and Cragg (3, p. 153-163). They included a fresh water form, G. pulex, a brackish water form, G. duebeni, and two marine species, G. locusta and G. obtusatus. G. locusta is sometimes found in brackish water. The authors were primarily

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[1] Since this term is often used in a confusing and misleading sense, clarification is necessary. Beadle (2, p. 173) used the term osmoregulation to imply "regulation of body fluid osmotic concentration." Robertson (28, p. 277), Jones (14, p. 80), Krogh (15, p. 5) qualified this definition by implying "regulation of body fluid osmotic concentration at levels different from the osmotic concentration of the environment." To avoid confusion the term osmoregulation, as used in this paper, may be defined as the maintenance of body fluid osmotic concentrations compatible with life, but differing from the osmotic concentration of the environment. Thus, the internal body fluid osmotic concentration may change, but if it remains different from the external osmotic concentration and the animal is able to survive, the animals are osmoregulating.

interested in the relation between external medium and the blood, when the salinity of the former was rapidly changed. It was found that the two marine species were not able to survive in a dilute medium, while the fresh water form was able to survive. The brackish water form was able to withstand both extremes. To explain this, Beadle (3, p. 164) postulated that while the tissues are in osmotic equilibrium with the blood there are ionic differences. He confirmed this by determining blood-tissue chloride differences, and was able to show that the brackish water form, G. duebeni, and the marine form, G. locusta, were able to withstand a greater range in tissue chloride than were the other two species.

Another group of crustaceans on which considerable work has been done are the semi-terrestrial crabs. The osmotic behavior of several of these crabs has been investigated by Gross (10, p. 205-222) and Jones (14, p. 79-92). The species studied were: Uca cenulata, which burrows near the high tide mark in bays; Pachygrapsus crassipes, and Hemigrapsis nudus both of which are found under rocks near or above the high tide mark. The latter two are also found in brackish water of bays. All three crabs show considerable hyperregulation[1] in dilute medium

[1] Hyperregulation denotes the ability of an organism to maintain its internal environment (blood) at a higher osmotic concentration than their external environment.

and Uca and Pachygrapsus show, in addition, hyporegulation[1] in a concentrated medium. In all cases the urine excreted remained isotonic to the blood.

Gross (11, p. 53) was able to show that degree of osmoregulation was correlated with the degree of exoskeletal permeability to salts and water. Better regulators (such as Pachygrapsus and Hemigrapsus) were relatively impermeable while non-regulators (such as Cancer and Pugettia) were highly permeable to both salts and water. Since the urine is isotonic to the blood, a decrease in exoskeletal permeability would necessitate active salt uptake through other boundary membranes in order to maintain a hyperosmotic condition in dilute sea water. By the same reasoning, in order to maintain a hypoosmotic body fluid in a concentrated medium, water would have to be actively taken up and salts excreted. There is some evidence that active ion or water uptake is accomplished through the gills and to some extent through the gut (8, p. 76-87; 7, p. 37-48).

The exact forces involved in causing Uca, Pachygrapsus and Hemigrapsus to develop hyper- and hypo-osmotic

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[1] Hyporegulation denotes the ability of an organism to maintain its internal environment (blood) at a lower osmotic concentration than their external environment.

regulation are obscure. These crabs are, in fact, semi-terrestrial, and the reasons for their developing this type of regulation may be due to their evolution towards land and not their invasion of fresh water. Gross (10, p. 210-222; 11, p. 60-62) points out that it is unlikely these crabs ever encounter anything but occasional osmotic stress to either dilute or concentrated environments. The reason that hyporegulation is exhibited in the laboratory may be because it involves the same mechanisms of active water uptake and salt excretion that are involved in water conservation. If this is the case, then brackish water invasion by these forms would be a secondary event following the partial invasion of land.

The invasion of fresh water has occurred, according to Beadle, in two main steps; both of which seem to allow the organism to survive successfully in fresh water (3, p. 55). These steps are: (1) a high blood osmotic concentration associated with a large blood tissue chloride gradient; and (2) evolution of renal salt re-absorption mechanisms and a lowering of blood concentrations and blood tissue chloride gradients to levels more easily maintained (3, p. 56).

Eriocheir, the Chinese wool handed crab, has taken the first step towards adaptation to fresh water. This crab can penetrate fresh water but must return to salt water to

breed. This invasion into fresh water, is accomplished by a reduction in permeability to both salts and ions. Thus, it may maintain a high blood concentration and still excrete a urine isotonic to the blood (21, p. 28).

The isopod Mesidotea entomon, a post glacial immigrant to fresh water, is similar to Eriocheir. Mesidotea is found living in full-strength sea water, and in the Baltic Sea in areas where the sea water is greatly diluted. The same organism is also found in fresh water lakes in Sweden. A comparison of the osmotic behavior of both the fresh and brackish water forms showed that they were different. The Baltic form is able to survive in 100 per cent sea water, but not in fresh water. The Swedish form can survive in both 100 per cent sea water and in fresh water. Thus, there is a clear physiological distinction between the two forms. The major difference in the osmoregulatory behavior of the two is that the fresh water form has developed a more effective osmoregulatory mechanism enabling it to maintain a high blood osmotic concentration in fresh water, similar, in fact, to that of the marine form (17, p. 256). It is not known whether the fresh water Mesidotea excretes an isotonic urine, or whether the animal has reduced its permeability to salts and water. One would expect that this animal behaves similarly to Eriocheir and has reduced its permeability and does excrete an isotonic urine.

Shaw (32, p. 157-176) found that Potamon miloticus, a fresh water crab in the rivers and lakes of Kenya, is a well established successful fresh water crustacean. This crab, however, resembles a typical fresh water animal only in one respect; namely, its inability to survive in sea water. When Potamon was subjected to various dilutions of sea water it was able to survive indefinitely only in concentrations not exceeding 50 per cent sea water. In 75 per cent sea water some animals survived for over three months but in 100 per cent sea water the crabs tested died within four days. Shaw found the osmotic concentration of the blood to resemble that of Eriocheir and Mesidotea except for being somewhat lower. He also observed that this crab was a hyper-osmotic regulator up to 100 per cent sea water where its hyper-osmotic regulation ceased. The urine secreted by Potamon was isotonic to the blood, even when the blood concentration was caused to increase by exposure to concentrated sea water. The invasion of fresh water by Potamon is made possible by its reduced permeability to both salts and water and, therefore, little salt is lost through the carapace. Salts are actively taken up which allows Potamon to survive in fresh water with a relatively high blood concentration and with the production of an isotonic urine. The same condition exists in Eriocheir but to a lesser degree (32, p. 174).

The fresh water prawn, Palaemonetes antennarius, from the Po Valley in Italy, survives fresh water invasion in a manner different than Potamon. Parry (19, p. 417-423) showed that this organism had a blood osmotic concentration equal to that of the fresh water crayfish. The urine produced, however, was isotonic to the blood, in contrast to the crayfish, which produces a hypotonic urine (16, p. 158; 26, p. 203-306; 32, p. 554; 13, p. 523). Furthermore, the urine production was calculated to be ten times greater than that reported in the crayfish Cambarus by Lienemann (16, p. 153). The fresh water prawn is apparently a successful fresh water animal but one incompletely adapted to its environment. It has met its new environment only by a reduction in blood concentration, and must live in fresh water presumably at considerable energy expense (19, p. 422).

The crayfish has achieved Beadle's second stage of fresh water adaptation; namely, a low blood concentration and the excretion of a urine hypotonic to the blood. The survival of crayfish in dilutions of sea water is similar to that of Potamon, already mentioned. Bogucki (4, p. 174) was able to keep Astacus fluviatilis, the European crayfish, alive for more than a month in 50 per cent sea water and for more than three months in 66 per cent sea water, but in 100 per cent sea water the crayfish did not survive. Slow

adaptation did not seem to increase the longevity of A. fluviatilis as they died shortly after being placed in 100 per cent sea water. Lienemann (16, p. 149-161) found that Cambarus clarkii did not survive well in an isotonic solution of sodium chloride and that acclimation did not help. This is of little value to the study of osmotic behavior, however, because she did not use sea water. The reason her crayfish died is probably due to some other ion not being present which would, if present, enable the tissues to function at the new osmotic blood concentration.

The adaptation of crayfish to fresh water is probably accomplished by a decrease in permeability to salts with a corresponding decreased permeability to water (32, p. 157). This would necessitate removal of large amounts of water and re-absorption of necessary ions by some mechanism. Riegal (26, p. 296-307) and Lienemann (16, p. 149-161) have shown that the antennal gland functions in this manner.

Little work has been done on the osmotic behavior of the North American crayfish. Thus, it was felt that a systematic study of the osmotic behavior in various dilutions of sea water was needed. It was felt that an Acute Experiment, in which the animals are subjected to a sudden osmotic stress, versus an Acclimation Experiment in which the animals are gradually adapted to various

salinities, might best elucidate the osmo-regulatory behavior of the test animals. Further, as mentioned earlier, several reports of crayfish from brackish water have been reported. George Miller (18, p. 123) reports finding crayfish with the barnacle Balanus improvisus on their carapaces. There are records of crayfish being caught in brackish water from the Youngs River, the Siuslaw River and the Lower Columbia River (18, p. 123). It was felt that these "brackish" water forms might differ, in respect to osmotic behavior, from true fresh water crayfish. Two populations were chosen, one from the Alsea River, because of its similarity to the Siuslaw River; and the other from Suttle Lake, because of its geographic isolation from the sea.

MATERIALS AND METHODS

Capture and Maintenance of Animals

Two widely separated populations of crayfish were chosen, one from the Alsea River and the other from Suttle Lake. Both populations were determined to be Pacifasticus leniusculus trowbridgii, on the basis of a key by George Miller (18, p. 144-147). The Alsea River crayfish were trapped about four-tenths of a mile down stream from the highway designation of Tidewater. Tidewater is 11 miles from the ocean, by highway (Oregon 34), and slightly further by the river. In August and September, when the crayfish were trapped, the river at this point was subjected to tidal fluctuations. Because of these fluctuations, the sea water content varied between 0 and about 10 per cent. The river here, however, is not effected by tidal fluctuations in the winter, due to the large amounts of "runoff" water carried by the river.

Suttle Lake is located near the summit of the Cascade Mountains, on the Santiam Highway (US 20) at an elevation of approximately 4500 feet. It is approximately 105 miles from the ocean. The crayfish were collected in October at the outlet of the Lake near the Suttle Lake Lodge.

Minnow traps baited with fish were used to capture the crayfish. They were brought in from the field and placed in shallow galvonized containers lined with fiber

glass. City water, filtered through charcoal to remove chlorine, was circulated through the tanks. The water was aerated at all times and the temperature maintained between 12 and 17° C. The animals were fed ground beef liver three times a week, and again 24 hours before an experimental run. Under the above conditions, crayfish were observed to live, without noticeable adverse effects, for as long as ten months.

Healthy male crayfish ranging in weight from 30 to 60 grams, were used in the experiments. Only intermolt animals were used. Baumberger and Olmsted (1, p. 531-544) found that crabs, during pre-molt and post-molt stage, showed abnormal osmoregulation. It was found by preliminary investigation (see Results) that crayfish showed a similar phenomenon. The intermolt stage was determined by the non-compressibility of the carapace and by the absence of a gastrolith.

Experimental Protocol

Two series of experiments were run on each population of Pacifasticus leniusculus trowbridgii. Both populations were subjected to the same experimental procedure.

In the first series of tests, termed the Acute Experiment, the animals to be tested were removed from the holding tank and placed in three liters of sea water of the desired salinity. This was obtained by first adjusting

ocean sea water to a concentration equivalent to 524 millimoles of sodium chloride per liter by the use of evaporated sea water or filtered city water. This was then designated as 100 per cent sea water, and diluted with filtered tap water to the desired concentration. Only one animal was placed in each container to avoid any effects of crowding. Dilutions of 0, 20, 40, 60, 80 and 100 per cent sea water were used. Due to the lethality of 100 per cent sea water it proved necessary to run this salinity separately. The animals were kept in the test solutions for 48 hours, after which both blood and urine were analyzed for osmotic concentration. It has been shown in certain other crustaceans that an equilibrium[1] is reached within 24 hours after being placed in the test solution (11, p. 47). Since nothing is known about the time it takes a crayfish to reach an equilibrium with its' external medium a survival test was run on 12 crayfish to see how long they could stand being stressed in 100 per cent sea water. It was found that within 48 hours only two of the 12 crayfish had died, but in 72 hours six had died. Forty-eight hours was chosen primarily because it was longer than the 24 hours reported by Gross

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[1] Equilibrium here does not mean that the body fluids are isotonic to the external medium, but that the blood of the animal has reached a steady state with respect to osmotic pressure.

(11, p. 47) and because it was the longest period of time in which the minimum number of animals died.

The second series of tests, termed the Acclimation Experiment, were designed to show the effects of acclimation on osmoregulatory behavior. The animals to be tested were taken from the holding tanks and placed in containers holding three liters of fresh filtered water. After 48 hours, three to four animals were sampled for blood and urine. The remaining animals were fed and one hour later were changed to containers of three liters of 20 per cent sea water. This procedure was repeated until 100 per cent sea water was reached, the total time for acclimation to 100 per cent sea water being 12 days.

Sampling Procedure

Crayfish were first washed with fresh tap water to remove all salt residue, and then blotted with a paper towel. The water in the branchial chamber was blown out, and the ventral surface dried, with a stream of compressed air. This entire procedure occupied less than three minutes. It was felt that speed was necessary to prevent any changes in blood concentration due to stress. There is, however, no evidence that this might occur in crayfish. The crayfish was then placed on a holding board, and held in place with rubber bands.

Blood was obtained by inserting a short piece of capillary tubing, (3 cm. long, 0.8 to 1.1 mm. in diameter) into the lateral sinus through the joint between the basipodite and coxopodite. The blood pressure was sufficient to cause the blood to "well" over the top of the capillary tube. The blood continued to flow out of the capillary for about 60 seconds. Samples for osmotic pressure determination were taken, as described in the following section, before the blood stopped flowing.

Urine samples were collected according to a modified method of Riegel (26, p. 297). The crayfish stores urine in a bladder, opening to the outside through a curved duct. The nephropore opening to the outside is guarded by a flap called the operculum. The urine was drawn from the nephropore by gently tickling the operculum with a capillary tube. According to Riegel (ibid.) this method obviates any danger of contamination by blood. As soon as the urine appeared it was picked up in a capillary tube for analysis.

Measurement of Osmotic Pressure

Samples for analysis were drawn into 75 mm. lengths of capillary tubing (.8 to 1.1 mm. in diameter) by placing a finger over one end and alternately dipping and withdrawing the tube from the fluid sampled until fluid filled about 2 mm. at the end of the tube. Thus each tube

contained about 0.002 ml. of fluid. The fluid was then drawn to the center of the tube with the aid of a 1 cc. syringe fitted with a number 30 needle. The needle was greased with petroleum jelly which offered a seal between the seat of the needle and the capillary. By gently withdrawing the syringe plunger in a rotary manner the sample could be centered in the tube. Whenever possible, duplicate samples were taken. Both ends of the capillary tube were then sealed off with petroleum jelly; care being taken not to force the vasoline in and thus cause a pressure increase within the tube. The samples were frozen until analyzed.

Osmotic concentration was determined by a freezing point method described originally by Jones (14, p. 81) and since modified by Gross (10, p. 405). This method is based on a comparison of the melting times, in a slowly warming brine bath, of frozen samples of the unknown and of sodium chloride solutions of known concentration. The measurements were carried out in a specially designed insulated melting point box consisting of an outer wooden box separated by four inches of styrofoam insulation from a four-liter capacity Lucite brine container. Lucite windows were placed on both the front and back of the box, so the observer could look through the brine bath. The window farthest from the observer was frosted to diffuse light. Two pieces

of polaroid plastic from the same sheet, but cut at right angles, covered the front and back windows respectively. A florescent light, placed behind the back window, allowed light to pass through the back polaroid, the brine and the front polaroid. Very little light managed to come through the crossed polaroids but when a frozen sample was placed in the brine it rotated the light causing the frozen crystals to be seen as a bright glow. This allowed for a sharp distinction between thawed and frozen samples. A stirrer was placed in the brine to insure an equal temperature distribution within the bath.

The samples to be analyzed were placed in a brass rack, constructed to hold 29 tubes. Only a few tubes were used if the unknowns were suspected to be of equal or near concentrations, which helped to prevent errors due to a small time lapse between tubes. Samples of known sodium chloride concentration were placed in the rack with the unknowns, and the samples quick-frozen in a dry ice-acetone slush. The sample rack was then placed in the brine bath, previously cooled to -10° C. The temperature of the brine was then brought up to about -2° C. with the aid of hot water. The time of melting in each capillary was recorded and plotted against those of known concentrations. The values of the unknown were then obtained by interpolation and expressed as equivalents of sodium chloride.

RESULTS

Effects of Molting

Two groups of crayfish from the Alsea River were tested. One group was tested in August and its members were considered to be in the intermolt condition. These animals will be compared later with the Suttle Lake crayfish. The other group was tested in May and its members were considered to be in the premolt or Stage D of the molt cycle, as originally described by Drach (cited by Scheer 29, p. 4). The animals were considered to be in Stage D whenever the carapace was easily depressible and when, on autopsy, the gastroliths were visible. Both groups were acutely tested and in every way treated similarly.

Table 1 and Figure 1 are a comparison of the total blood osmotic concentration of the two groups tested. The broken line in Graph 1 represents the crayfish in "D Stage." The solid line represents crayfish in the intermolt stage. The circles, in both cases, represents the mean blood osmotic concentration of from four to six animals (Table 1). The vertical lines above and below the circles represent plus and minus one standard error.

There are no controls (animals from fresh water) for the Stage D animals. The blood samples were inadvertantly destroyed and when the mistake was discovered the animals

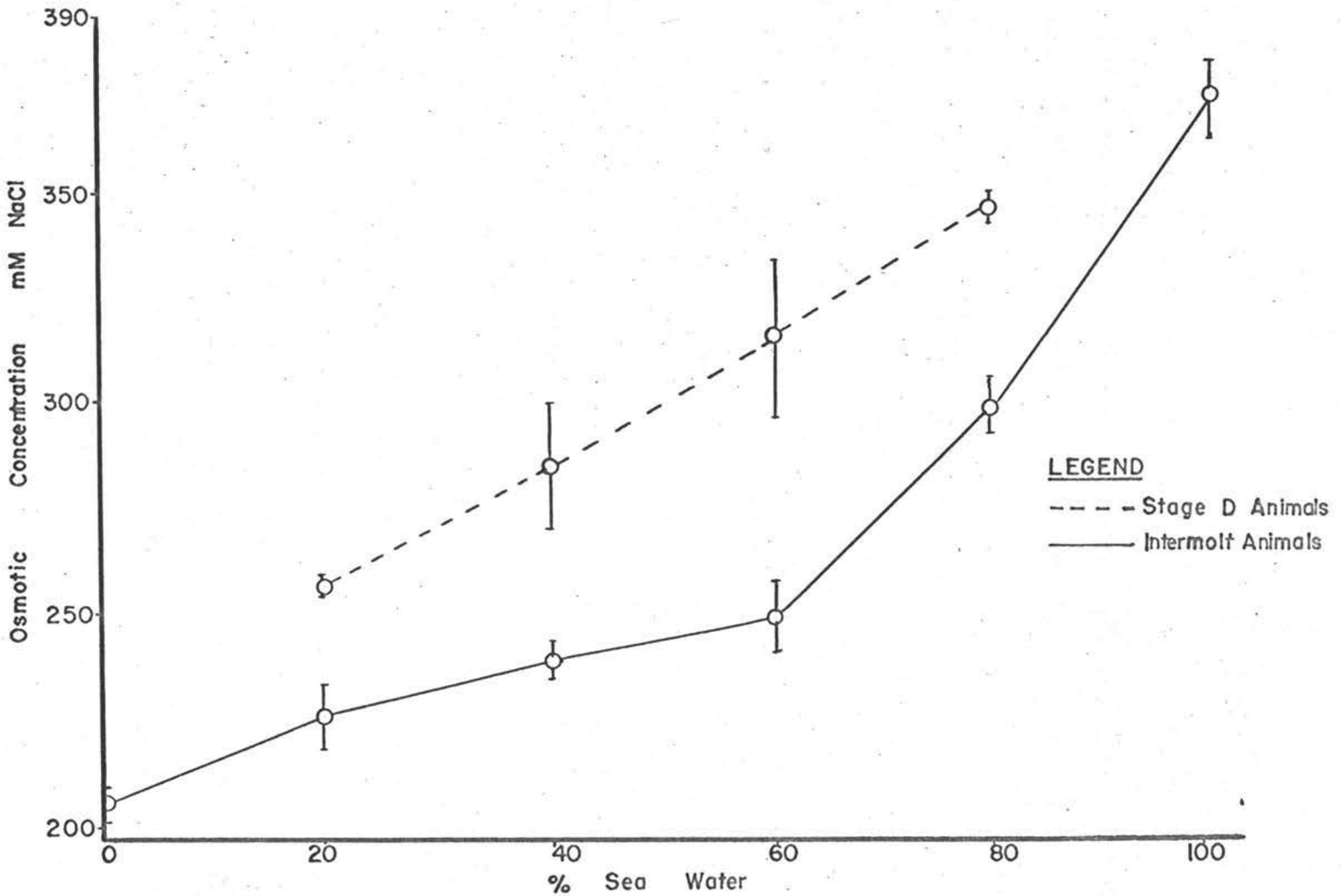

Figure 1. COMPARISON OF STAGE D AND INTERMOLT CRAYFISH FROM THE ALSEA RIVER.

Table 1. COMPARISON OF BLOOD OSMOTIC CONCENTRATIONS (EQUIVALENT TO mM NaCl ± 1 STANDARD ERROR) OF PREMOLT AND INTERMOLT CRAYFISH.

Animals Taken from the Alsea River.

	Premolt		Intermolt*	
Percent Sea Water	No. of Animals	Blood Osmotic Concentration	No. of Animals	Blood Osmotic Concentration
0	0		7	205±5
20	6	257±1	7	226±8
40	5	285±15	5	239±5
60	6	315±19	7	249±9
80	4	348±2	5	298±9
100	-	---	7	372±8

* Data also shown in Table 3.

in the Alsea River had completed their molt and Stage D animals could not be found.

Acute Experiment

The animals in this experiment were subjected to the test solutions for 48 hours. Blood and urine were then sampled and tested for total osmotic concentration and reported as millimoles equivalence of sodium chloride. Test solutions of 0, 20, 40, 60, 80 and 100 per cent sea water were used. One hundred per cent sea water was considered to be equal to an osmotic concentration of 524 millimoles of sodium chloride (mM of NaCl). The result of the blood and urine measurements for the Acute Experiments are summarized in Figure 2 and Tables 2 and 3.

The blood osmotic concentration of the Suttle Lake animals remains at a constant level of about 240 mM of NaCl when the animals are tested in 0, 20, 40 and 60 per cent sea water (fig. 2). The standard errors are such that a straight line may be drawn from the control or 0 per cent treatment, through the 20 per cent and 40 per cent treatment values without falling outside plus or minus one standard error. Thus, the Suttle Lake crayfish are considered to maintain a constant internal osmotic state when subjected to salinities up to 60 per cent sea water. From this point the crayfish can no longer maintain its internal osmotic concentration and it begins to rise,

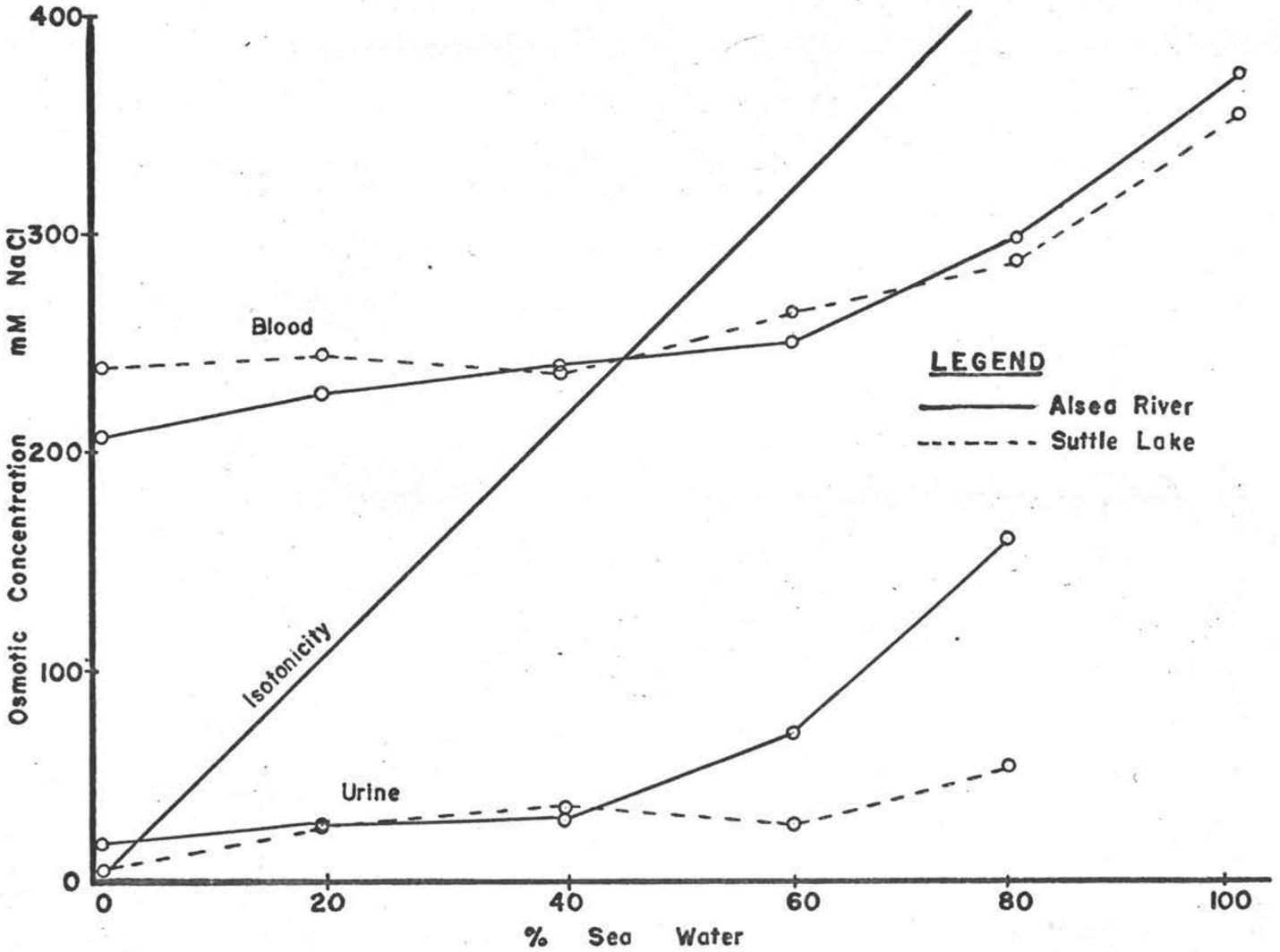

Figure 2. ACUTE EXPERIMENT--COMPARISON OF THE ALSEA RIVER AND SUTTLE LAKE POPULATIONS.

Table 2. ACUTE EXPERIMENT. SUTTLE LAKE CRAYFISH.

Mean blood and urine values with standard errors, reported as equivalent to mM of NaCl.

Treatment Per Cent Sea Water	Blood		Urine	
	No. of Crayfish Sampled	Osmotic Concentration (mM NaCl)	No. of Crayfish Sampled	Osmotic Concentration (mM NaCl)
0	8	237±6	8	6±1
20	8	244±6	8	25±8
40	7	237±5	7	35±8
60	8	262±9	8	26±6
80	8	285±10	6	53±20
100	8	354±8	-	---

Table 3. ACUTE EXPERIMENT. ALSEA RIVER CRAYFISH.

Mean blood and urine values with standard error, reported as equivalent to mM of NaCl.

Treatment Per Cent Sea Water	Blood		Urine	
	No. of Crayfish Sampled	Osmotic Concentration (mM NaCl)	No. of Crayfish Sampled	Osmotic Concentration (mM NaCl)
0	7	205±5	7	18±7
20	7	226±8	6	24±4
40	5	239±5	6	29±4
60	7	249±9	7	68±17
80	5	298±8	5	159±25
100	7	372±8	-	---

reaching a maximum of 354 mM of NaCl in 100 per cent sea water.

The osmotic concentration of the urine of the Suttle Lake animals remained decidedly lower than that of the blood. The urine osmotic concentration of animals in 20 per cent sea water was slightly higher than the fresh water controls (fig. 2). Between 20 and 80 per cent sea water the urine osmotic concentration remained essentially constant. In 80 per cent sea water, urine concentration increased to 53 millimoles of sodium chloride, a value still decidedly hypotonic to the blood. No urine was obtained from animals in 100 per cent sea water because of drastic reduction in urine flow under these conditions.

The mean blood osmotic concentration of the Alsea crayfish increased gradually up to 60 per cent sea water (Figure 2, Table 3). Between 20 per cent and 60 per cent sea water, the blood is almost identical with the Suttle Lake blood. In 60 per cent and 80 per cent sea water, blood osmotic concentration shows a more pronounced increase: to values somewhat higher than those from Suttle Lake (fig. 2).

Urine concentration is almost identical to that from Suttle Lake forms up to 60 per cent sea water (Table 2, Figure 2). In 60 and 80 per cent sea water, there is a dramatic increase in urine concentration, the value for

animals in the highest salinity stress being more than one half that of the blood (Table 2).

Acclimation Experiment

The animals in this experiment were first placed in filtered fresh water and then transferred after 48 hours into containers of 20 per cent sea water. Each 48 hours this procedure was repeated until the maximum salinity had been reached. Blood and urine samples were analyzed at 0, 20, 40, 60, 80 and 100 per cent sea water. As in the previous experiment 100 per cent sea water was equal to 524 mM of NaCl. The results of the measurements of blood and urine total osmotic concentrations are summarized in Figure 3 and Tables 4 and 5.

Blood osmotic concentrations from the Suttle Lake animals in 0 and 20 per cent sea water were nearly identical to the values from the Acute Experiment, as would be expected since the acute and acclimated crayfish were handled the same way at these salinities. Mean values then increased gradually and uniformly to the maximum of 370 mM of NaCl in 100 per cent sea water, a value only slightly higher than the maximum in the Acute Experiment (354 mM of NaCl).

The urine from the acclimated Suttle Lake crayfish at salinities from 0 to 40 per cent was similar in osmotic concentration to that excreted by the Suttle Lake animals

Figure 3. ACCLIMATION EXPERIMENT--COMPARISON OF THE ALSEA RIVER AND SUTTLE LAKE POPULATIONS.

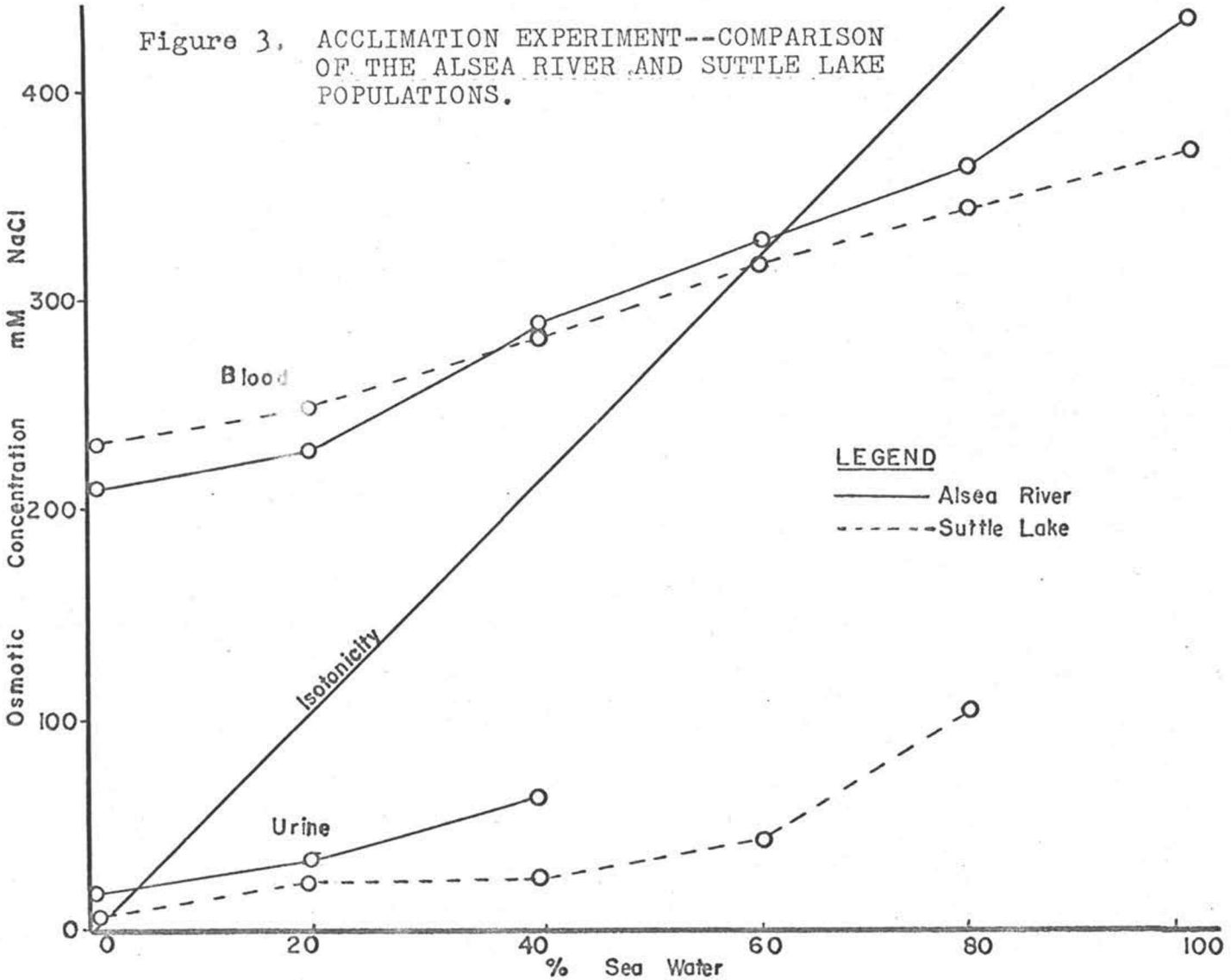

Table 4. ACCLIMATION EXPERIMENT. SUTTLE LAKE.

Mean blood and urine values with standard error, reported as equivalent to mM of NaCl.

Treatment Per Cent Sea Water	Blood		Urine	
	No. of Crayfish Sampled	Osmotic Concentration (mM NaCl)	No. of Crayfish Sampled	Osmotic Concentration (mM NaCl)
0	8	232±2	8	6±1
20	7	248±9	7	22±14
40	10	281±25	8	24±6
60	10	318±7	7	41±5
80	11	341±8	7	94±21
100	10	370±8	-	---

Table 5. ACCLIMATION EXPERIMENT. ALSEA RIVER.

Mean blood and urine values with standard error, reported as equivalent to mM of NaCl.

Treatment Per Cent Sea Water	Blood		Urine	
	No. of Crayfish Sampled	Osmotic Concentration (mM NaCl)	No. of Crayfish Sampled	Osmotic Concentration (mM NaCl)
0	6	211±8	6	18±7
20	6	229±5	6	31±5
40	6	291±4	5	63±5
60	6	328±14	2	70
80	6	362±5	-	---
100	6	433±4	-	---

in the Acute Experiment. The urine from the animals acclimated to 60 and 80 per cent sea water is higher in concentration than the urine from animals acutely stressed. The amount of urine produced was less at these higher salinities.

The blood osmotic concentrations of the acclimated Alsea River crayfish in 0 and 20 per cent sea water were, again, almost identical to the values from the acute curve (fig. 2). In 40 per cent sea water osmotic concentration increases to a value higher than that of the Suttle Lake forms. The Alsea curve consistently remains above the Suttle Lake curve at the higher salinities, with an especially pronounced increase in blood concentration from 80 to 100 per cent sea water.

The osmotic concentration of the urine of Alsea animals sampled at 20 per cent varies little from the control animals. Those animals sampled at 40 and 60 per cent had a more concentrated urine than the corresponding animals in the Acute Experiment. The volume of urine produced in the 60 per cent animals was so low, however, that only two samples could be taken from six animals. No urine could be obtained from animals in 80 per cent and 100 per cent sea water.

DISCUSSION

Before the results from the two populations can be discussed, other pertinent aspects which have a direct bearing on these results must be examined. The comparative study was carried out over an eight month period. The Alsea River Acute Experiment was done in August and September. The remaining experiments were done in January, February and March. It was felt that there could be fluctuations, of a seasonal nature, in the osmotic behavior of the crayfish studied and that these fluctuations, if present, would have a bearing on the conclusions to be drawn from the results.

The most obvious seasonal phenomenon in crayfish is molting. According to Miller (18, p. 121) the adult crayfish, Pacifasticus leniusculus trowbridgii experiences two molts per year, one during April and May and the other during August and September. The crayfish from the Alsea River used in the Acute Experiment were trapped before the August and September molt. Animals in Stage D of the molt cycle were not found with this group of crayfish. The Alsea River crayfish used in the Acclimation Experiment were trapped before the onset of the April-May molt. Some of these crayfish went into the Stage D in May and were used in a comparison of the intermolt and Stage D

animals (fig. 1). The animals taken from Suttle Lake were trapped after the August-September molt. These animals possessed what appeared to be new exoskeletons free of ectoparasites, normally found present on older exoskeletons. However, the exoskeletons were hard and, therefore, these crayfish were considered to be intermolt animals. The Suttle Lake crayfish which were not used in the experiments did not reach Stage D of the molt cycle until the following June. A comparison of the Alsea River intermolt and the Stage D animals (table 1, fig. 1) shows an apparent difference between the osmotic behavior of the two groups when acutely tested. Baumberger and Olmsted (1, p. 531-554) have shown that in grapsoid crabs the osmotic concentration of the blood also increases prior to molting and returns to normal shortly after molting. Thus, animals suspected to be in a premolt condition were not used in the tests comparing the two populations.

Examination of the curves of blood osmotic concentration (figs. 2 and 3) indicate that the two populations show the same trends when exposed to various dilutions of sea water. Under the conditions of acute stress (fig. 2, tables 2 and 3) Pacifastacus shows hypertonic regulation when placed in a medium less concentrated than the blood and hypotonic regulation when placed in a more concentrated medium. Blood osmotic concentration remains at a relatively constant

level up to 60 per cent sea water. In higher salinities it begins to increase, the most pronounced rise occurring between 80 and 100 per cent sea water (fig. 2, tables 2 and 3) where osmotic regulation apparently begins to break down. In the Acclimation Experiment (fig. 3, tables 4 and 5) where the animals were gradually brought up to full strength sea water, the blood osmotic concentration shows a gradual increase, in both populations, over the entire range of salinities tested. Between 80 and 100 per cent sea water, however, the increase in blood concentration of the Alsea River animals was much greater than that of the Suttle Lake animals. In 100 per cent sea water the difference in mean blood osmotic concentration between the two populations was greater than three times the sum of the standard errors of the mean[1].

Very little systematic work has been done on the osmotic regulation of crayfish, most studies being concerned with ionic regulation. Lienemann (16, p. 152) found that when Cambarus clarkii was exposed for 48 hours to a medium isotonic with its blood (about 33 per cent sea water) the

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[1] In this paper standard errors are used in the following way to denote differences between population samples: If additional samples were obtained their means would fall within plus or minus one standard error 68.27 per cent of the time, within plus or minus 2 standard errors 95.73 per cent of the time, and within plus or minus three standard errors 99.73 per cent of the time (5, p. 371).

blood osmotic concentration increased from its normal value equivalent to 184 mM of NaCl to a value equivalent to 290 mM of NaCl, indicating some breakdown in osmoregulation. Prosser (22, p. 18) makes the statement, presumably based on the above studies by Lienemann (16, p. 152) that crayfish cease to regulate blood osmotic concentration when the external medium is equal in concentration to the internal medium. The present study, and in particular the Acute Experiment, indicate that Pacifastacus is a better osmoregulator than Cambarus. It is important to note, however, that at all salinities above 20 per cent sea water, the animals in the Acclimation Experiment had higher blood osmotic concentrations than those in the Acute Experiment. This would indicate that 48 hours was not a long enough time for the blood to come into equilibrium with the external medium, and that if a longer exposure time had been chosen for the Acute Experiment, this curve would probably have approached the acclimation curve.

The urine values obtained in the two experiments (figs. 2 and 3, tables 4 and 5) indicate that the urine remains markedly hypotonic to the blood up to 60 per cent sea water. In 80 per cent sea water the urine osmotic concentration increases, albeit in a highly variable fashion, which could reflect the increased blood concentrations at these salinities. However, the largest increases in blood

concentration occurred, in both experiments, between 80 and 100 per cent sea water; but unfortunately, no urine samples could be obtained from any animals subjected to 100 per cent sea water. In fact, for the Alsea River acclimated crayfish, in 60 per cent sea water, only two urine samples could be collected from six animals, and no urine was collected from animals in higher salinities. The difficulty in collecting urine is due to the great reduction in urine production in hypertonic environments, a phenomena also observed in crayfish by other investigators (31, p. 554; 13, p. 523; 16, p. 155; 4, p. 174). The small number of animals involved, the great variability in the urine concentration values, and the absence of information on the rate of urine production, makes further discussion appear unjustified.

Although the two populations studied behave similarly, on the whole, when exposed to osmotic stress, there is an apparent difference in the osmotic concentration of the blood of the control animals (zero per cent sea water). The Alsea River crayfish have a blood osmotic concentration equivalent to 205±5 standard error (S.E.) mM of NaCl, (Table 3) while the Suttle Lake crayfish have a blood osmotic concentration equivalent to 237±6 (S.E.) mM of NaCl (Table 2). The variability between individual animals in both cases is small as indicated by the small

standard errors. Furthermore, the difference between the two populations is greater than three times the sum of the standard errors of the means. If one combines the means of the control animals from the Acute Experiment with the corresponding means of the control animals of the Acclimation Experiment (Tables 4 and 5), a larger number of animals is represented. If this is done the mean blood osmotic concentration from the Alsea River population is equivalent to 207±4 (S.E.) mM NaCl and the mean blood osmotic concentration for the Suttle Lake population is equivalent to 234±3 (S.E.) mM of NaCl. The difference between the two populations is still three times the sum of the standard errors. There seems to be no adequate explanation for the fact that the lower blood osmotic concentration is found in those animals living in an environment where they are frequently subjected to dilutions of sea water.

The urine osmotic concentration values obtained from the control animals tend to bear out the differences in blood concentration, i.e., the urine of the Alsea River animals is consistently higher in osmotic concentration, which could mean that salts are being lost from the blood, thus lowering the blood osmotic concentration.

Qualitative observations on the blood further supports the idea that there might be a real difference in the blood

of the two populations. The blood from the Alsea River animals was reddish in color, opaque, and contained a large quantity of formed elements. Blood from the Suttle Lake animals was clear, light blue, contained few formed elements, and was not as viscous as that from the Alsea River animals. Although these observations can hardly be considered quantitative they suggest that further study of the morphology of the blood of these two populations might prove rewarding.

In comparing the normal fresh water blood values with those of other crayfish, the Alsea River population is found to resemble most closely Cambarus clarkii studied by Lienemann (16, p. 149-161) and the Suttle Lake population resembles Astacus trowbridgii, studied by van Harreveld (33, p. 428). It is suspected by the writer that the latter is the same animal that is now called Pacifastacus leniusculus or at least a closely related species. Table 6 gives the normal blood concentration values for a number of crayfish, together with the investigator.

A slight, but possibly significant, difference between the two populations, as expressed by the blood values, is seen in the animals exposed to 20 per cent sea water. The difference between the means is slightly less than twice the sum of the standard errors. This is thought

Table 6. A COMPARISON OF NORMAL BLOOD OSMOTIC CONCENTRATION VALUES OBTAINED BY A NUMBER OF DIFFERENT WORKERS.

Crayfish	Osmotic Concentration of Blood: Freezing Point Depression ° C.	Osmotic Concentration of Blood: Equivalent to mM NaCl*	Investigator
Cambarus clarkii	.64	184	Lienemann (16, p. 159)
Cambarus clarkii	.76	217	van Harreveld (33, p. 428)
Cambarus clarkii	.68	194	Schlatter (30, p. 259-261)
Astacus trowbridgii	.83	237	van Harreveld (33, p. 428)
Astacus fluviatilis	.80	228	Hermann (13, p. 523)
Astacus fluviatilis	.81	231	Scholles (31, p. 554)

* These values were calculated from the freezing point depression by assuming that one degree centegrade is equal to 285 mM of NaCl (24, p. 57-64).

to merely represent an extension of the same difference in osmotic concentration shown by the controls.

In the higher salinities (80 and 100 per cent sea water) the two populations showed differing abilities to survive a 48 hour acute stress. Of seven Alsea River animals subjected to 80 per cent sea water only five survived. Out of 22 tested in 100 per cent sea water, only seven survived the 48 hours. The Suttle Lake crayfish on the other hand, showed no mortality in either 80 or 100 per cent sea water. All the crayfish which survived 100 per cent sea water (from both populations) were inactive and the areas between the joints of the carapace and legs had a wrinkled appearance, indicating dehydration. None of these characteristics appeared in crayfish tested in salinities less than 80 per cent sea water. It is difficult to assess the adaptive significance of the different survival figures of the two populations in 80 per cent and 100 per cent sea water. Since one might expect the Alsea, rather than the Suttle Lake, animals to survive best under this stress, we can only suggest that perhaps the Suttle Lake animals were in better overall physiological "condition." It is interesting that in the Acclimation Experiment, no animals of either population died in 80 or 100 per cent sea water, indicating that gradual acclimation to these high salinities erases any

inherent difference in tolerance between the two groups. Extensive survival tests should be conducted to clarify this point.

The Alsea River crayfish chosen for this comparison were thought to represent a brackish water population (see Introduction). Alsea River crayfish used in this study are normally subjected to salinities up to at least 10 per cent sea water. Whether they are present in areas of greater salinity could not be determined. However, it is evident from the blood work and the survival data that they can not stand a sudden "acute" stress of 80 to 100 per cent sea water for a 48 hour period. On the other hand, they seem to survive very well if they are slowly acclimated to these higher salinities, even though their blood osmotic concentrations rise above the level found in the animals that were "acutely" stressed (Figure 3, Tables 4 and 5). This suggests that one of the physiological differences between the Alsea River crayfish and the Suttle Lake crayfish is the ability of the Alsea River crayfish to adapt its tissues by slow acclimation, to a high osmotic concentration. It has been shown in a number of marine and brackish water forms that adaptation of tissues to higher osmotic concentrations is possible (2, p. 173; 34, p. 483-486; 35, p. 378-385). As is shown in Figures 2 and 3, both populations behave similarly up

to 20 per cent sea water, but then the osmotic concentration of the blood of Alsea River crayfish rises to a level above the Suttle Lake forms. Therefore, if the Alsea River forms were ever to encounter high salinities normally, then the adaptation of their tissues is truly of survival value. It has been suggested that osmoregulation is an energy requiring process (20, p. 618-630), and therefore, it would require less energy to survive in more concentrated salinities if the blood osmotic concentration were allowed to approach the osmotic concentration of the environment.

Admittedly, the above explanations are highly speculative. More work needs to be done on the ecological distribution of the Alsea River crayfish. Also, a study of the ionic regulation of both the Alsea River and the Suttle Lake crayfish as well as studies of survival at both high and low salinities would help to elucidate possible differences between these populations.

SUMMARY

1. The osmotic regulation of two populations of fresh water crayfish, Pacifastacus leniusculus trowbridgii, was studied. One population was from Suttle Lake and the other population was from the Alsea River. Two sets of experiments were performed: an Acute Experiment and an Acclimation Experiment.
2. The osmotic concentration of the blood and urine was determined by the use of melting time techniques.
3. It was found that the Alsea River crayfish have a lower blood osmotic concentration than do the Suttle Lake forms. When stressed to higher salinities, either acutely or by acclimation, the order is reversed and the Alsea River crayfish have a higher blood osmotic concentration.
4. It was noted that the Alsea River crayfish show better survival when acclimated. This, coupled with their high blood osmotic concentration at 80 and 100 per cent sea water, suggests that they have the ability to adapt their tissues to a high osmotic concentration.

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