2007

THEOSMOREGULATORY ABILITIES OF FRESH WATER CRABS FROM THAILAND (POTAMIDAEANDPARATHELPHUSIDAE) AND BIOGEOGRAPHICALIMPLICATIONS

Lara Jin Qiu-ting Esser

THE OSMOREGULATORY ABILITIES OF FRESHWATER CRABS FROM THAILAND (POTAMIDAE AND PARATHELPHUSIDAE) AND BIOGEOGRAPHICAL IMPLICATIONS

By

Lara Jin Qiu-ting Esser

THESIS

Submitted to
Northern Michigan University
In partial fulfillment of the requirements
For the degree of

MASTER OF SCIENCE

Graduate Studies Office

2007

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

SIGNATURE APPROVAL FORM

This thesis by Lara Jin Qiu-ting Esser is recommended for approval by the student's thesis committee in the Department of Biology and by the Dean of Graduate Studies.

_____

Committee Chair: Dr. Neil Cumberlidge Date

_____

First Reader: Dr. Robert Winn Date

_____

Second Reader: Dr. Jacqueline Bird Date

_____

Department Head: Dr. Neil Cumberlidge Date

_____

Dean of Graduate Studies and Research: Dr. Cynthia Prosen Date

OLSEN LIBRARY
NORTHERN MICHIGAN UNIVERSITY

THESIS DATA FORM

In order to catalogue your thesis properly and enter a record in the OCLC international bibliographic data base, Olson Library must have the following requested information to distinguish you from others with the same or similar names and to provide appropriate subject access for other researchers.

NAME: Esser, Lara Jin Qiu-ting

DATE OF BIRTH: October 23, 1982

ABSTRACT

The osmoregulatory ability of four species of true freshwater crabs belonging to the Parathelphusidae (*Esanthelphusa dugasti* and *Sayamia germaini*) and the Potamidae (*Potamon smithianum* and *Larnaudia chaiyaphumi*), collected from four different localities in Thailand were investigated to test biogeographical hypotheses that depend on oceanic dispersal. Crabs were grouped by species, gender, and age and exposed to a range of salinity regimes from 0 to 30 ppt for up to two weeks in both deep and shallow water. Adult hemolymph osmolality was measured after 5, 9 and 13 days into the experiments, while juvenile hemolymph osmolality was measured at the conclusion of the experiment. Measured hemolymph and external water osmolality were used to determine if crabs were osmoregulating or osmosconforming in salinities ranging from fresh water to sea water. All four species were found to be active osmoregulators, and all survived in salinities up to 30 ppt for at least 5 days, and often much longer. The parathelphusid *Esanthelphusa dugasti* had the lowest mean hemolymph osmolality (373 mOsm/kg) of all four species, and no significant difference between males and females was found in the survival and osmoregulatory response.

Very small recently hatched potamids seemed to survive best in high salinities compared to adults and juveniles of the same species. Water depth influenced hemolymph osmolality only during the initial days of the experiment.

These results indicate that it is reasonable to believe that true freshwater crabs are capable of surviving for up to two weeks in full strength sea water which would be long enough to survive while crossing short distances between continents and nearby islands.

ACKNOWLEDGMENTS

I wish to thank my thesis advisor Dr. Neil Cumberlidge for all his help and knowledge and for conceiving of the idea for this project. This investigation gave me the wonderful opportunity to travel to Thailand and to work on living specimens of freshwater crabs that I was able to collect from their natural habitat in rainforests, waterfalls and rivers.

I would like to thank Dr. Robert Winn for being a wonderful professor, Dr. Jacqueline Bird for being a great resource for all kinds of questions, and Dr. Alan Rebertus for always being very helpful answering statistical questions.

I would like to thank Northern Michigan University for the Excellence in Education grant, and the Biology Department for awarding me the Development Fund grant. The research in Thailand was funded by an NSF grant awarded to Dr. Neil Cumberlidge.

I would also like to acknowledge the help of Jing Fang Niu, for being so patient and helping me to order all the supplies I needed as well as Suzie Piziali, without whom I would not have been able to fill out all those forms!

I am very grateful to Dr. Roger F. Uglow from the University of Hull, UK, who initiated my interest in Decapod crustaceans and who had the contacts which made it possible for me to do research at the Biology Department of Burapha University in Thailand.

Pongrat Dumrongjatawana kindly allowed me to work in his lab at Burapha University and helped organize the field trips to collect crabs. Dr. Nongnud is thanked for

allowing me to use the osmometer in her lab. In addition, Mr. Anuchard Kuhnsook kindly invited the collection team to stay at his house and to catch crabs on his fruit plantation at night. Kate (Burapha University) and Nobu are thanked for organizing and catching the rice field crabs (*Esanthelphusa dugasti*) in Chonburi. Finally Anuwat and his friends are thanked for helping me with all kinds of daily lab tasks and collecting the freshwater crabs during the field trips, and Chansawang helped to organize my stay beforehand.

This thesis follows the format of The Raffles Bulletin of Zoology.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

BW = brackish water
cw = carapace width
cl = carapace length
F = female
FW = fresh water
FWC = freshwater crab
J = juvenile
M = male
mya = million years ago
n = number
ppt = $^{o}/_{oo}$ = parts per thousand
RR = relative risk
SW = sea water

INTRODUCTION

1.1. Freshwater crab distribution and biogeography

Freshwater crabs (Potamoidea, Gecarcinucoidea, Pseudothelphusoidea) are brachyurans ('true crabs') that belong to the crustacean order Decapoda (Martin & Davis, 2001). Freshwater crabs differ from their marine crab relatives in that the former are restricted to fresh water habitats during all the stages of their life cycle. All true freshwater crabs exhibit direct development whereby all larval stages are lacking, so that their eggs hatch directly into juvenile (hatchling) crabs. In comparison to marine crabs, freshwater crabs are k-selected with a low fecundity, produce a small number of large eggs (e.g., 200 to 500 at a time), and provide parental care whereby hatchling crabs initially remain attached to the female pleopods in the abdominal brood pouch (Cumberlidge, 1999).

The global distribution of the freshwater crabs is circumtropical and they are found in South America, Central America, the Caribbean, Africa, Madagascar, southern Europe, parts of the Middle East and Near East, the Seychelles, India, Southeast Asia, China, the Philippines, Indonesia, New Guinea, and northern Australia (Sternberg & Cumberlidge, 2001). The distribution of freshwater crabs is in turn limited by their lack of the highly dispersive planktonic larval stages and by the restriction of these crustaceans to inland freshwater habitats. As a consequence, populations of freshwater crabs become reproductively isolated relatively easily, with the result that relatively small geographic areas often support a number of different species (Sternberg & Cumberlidge, 2001).

It has long been assumed that true freshwater crabs are unable to tolerate prolonged exposure to sea water, because these crabs are today restricted to freshwater habitats and are never found in either brackish water or sea water. This assumption raises the question of how freshwater crabs came to be distributed so widely on different continents and islands if they cannot survive in saltwater.

The origins of the freshwater crabs as a group are important in this context, but their sparse fossil record means that the age of the group is still uncertain. Vicariance hypotheses require an early (200 – 120 mya) origin for the group and assume the presence of freshwater crab ancestors either on Pangaea or Gondwana. This scenario assumes that these crabs dispersed throughout continental freshwater systems before the super-continents of Pangaea or Gondwana fragmented. Alternatively, if the group arose more recently (mid-late Cretaceous, 100 – 80 mya) at a time after the super-continents had fragmented then questions are raised as to how freshwater crab ancestors were able to disperse across the oceans to reach their current global distribution. Other questions concern the number of times that marine crab ancestors of freshwater crabs invaded freshwaters – was it only once, only a few times, or multiple times? If freshwater crab ancestors invaded freshwaters only once, or only a few times, then how did these freshwater animals disperse further across saltwater barriers to reach different continents and islands? Daniels et al. (2006) and Klaus et al. (2006) both assumed that freshwater crabs lack any tolerance to salt water, but disagreed on how many times the common ancestor of fresh water crabs invaded freshwaters, and how far crabs migrated thereafter.

The hypothesis of Ng & Rodriguez (1995) assumed sea water to be an absolute barrier to freshwater crab dispersal and viewed the freshwater crabs as an ancient group

that was present on Gondwana before the fragmentation of the continent. These workers argued that it was the breakup of this landmass that provided the passive means by which the ancestral freshwater crabs came to reach their present positions on the continents and islands (Rodriguez, 1986; Ng & Rodriguez, 1995; Ng et al., 1995). A competing hypothesis suggested that "various freshwater crab lineages (except trichodactylids) are not archaic but had a more recent (post-Cretaceous) derivation from a morphologically and phylogenetically advanced group of brachyurans" (Sternberg et al., 1999). The latter authors postulated that freshwater crabs originated from a widespread predominantly littoral marine crab clade, members of which independently invaded freshwater systems on different continents and islands along the shores of the Tethys Sea. This post-Gondwanan-fragmentation hypothesis assumed freshwater crabs to be a recent group. It was suggested that members of an ancestral circumtropical marine crab clade that was capable of surviving low salinity environments entered the freshwaters of all of the major continental landmasses and subsequently dispersed within the inland waters. Importantly in the context of the present work, some freshwater crabs may have crossed short distances over saltwater barriers to reach nearby islands at different times in the past.

The post-Cretaceous origins of freshwater crabs would have taken place during a period when the continents were in different locations compared to their present positions. For example, continental Southeast Asia is geologically an assemblage of small and large continental blocks (terranes) that rifted off the northern margin of Gondwanaland in the Devonian (400 mya), and continued to rift during the Early-Middle Permian, Late Triassic and Late Jurassic, before becoming part of the main Laurasian landmass (Protochina) in the Cenozoic (Mey, 1998; Metcalfe, 1998). During the Eocene

(50 mya), the continental block that included the Indian subcontinent collided with Asia, causing mountain building and major changes in climate habitats and drainage systems. These continental upheavals promoted the dispersal of Gondwanan organisms from India into Southeast Asia and created mountainous barriers between Southeast Asia and the rest of Asia. Another major geological event that affected the Asian fauna occurred in the Cenozoic (25 mya) when the northward drifting Australian microcontinental fragments made contact with Sulawesi, providing a possible pathway for the exchange of fauna and flora between Asia and Australia, while at the same time creating new barriers to dispersal (Hall, 1998).

The present work focuses on the freshwater crabs of Thailand, a country that geologically consists of two continental blocks or microcontinents. Eastern Thailand is part of the Indochina block while western Thailand (including the southern peninsula) is part of a different terrane. The timing of the collision and fusion of these two terrane blocks is debated and could have been as early as the Late Permian to Triassic (~ 250 mya) or as late as the Late Jurassic (~ 146 mya) (Metcalfe, 1998). In any event, at the time when the ancestors of freshwater crabs were supposed to have left the oceans and invaded freshwater (during the late Cretaceous, 80 – 65 mya) (Daniels et al. 2006), Thailand was already a single continental plate located at the margin of the Ceno-Tethys Sea.

Klaus et al. (2006) hypothesized that the mainly Asian gecarcinucoid freshwater crabs had a center of origin in Africa and from there migrated to Asia via Madagascan Lemurian island “stepping stones” in the Indian Ocean. Those authors further argued that falling seawater levels during the Pliocene (5.3 to 1.8 mya) exposed continuous land

bridges that enabled these freshwater crabs to move by land dispersal east from India to the Malesian archipelago and to the adjacent island groups (which were at that time a single landmass) without crossing any seawater barriers. Klaus et al. (2006) also argued that low sea levels during the Pleistocene (1.8 mya to 10,000 years ago) exposed land bridges that enabled freshwater crabs to further disperse over land to New Guinea (either via the Philippines or Sulawesi). Finally, those authors proposed that gecarcinucoid crabs entered the inland waters of the Australian mainland from New Guinea by means of a terrestrial connection throughout the Neogene (starting 23.8 mya) and Quaternary (starting 1.8 mya). Interestingly, Klaus et al. (2006) allowed for some tolerance of seawater to explain how the continental island freshwater crab genera *Seychellum* (on the Seychelles between Africa and India) and *Spiralothelphusa* (Sri Lanka) came to be on these islands.

To better understand the origins and dispersal of the freshwater crabs, biogeographical hypotheses can be tested by reference to the phylogenetic relationship within the group as a whole. The age of the group from fossil evidence would also be useful, but unfortunately, the fossil record for freshwater crabs is poor (Szombathy, 1916), with the earliest fossil freshwater crabs dating from the Miocene (25-30 mya) (Glaessner, 1930, 1969; Bott, 1955). A fossil of freshwater crab similar to the present-day *Potamon ibericum* from deposits in Austria was estimated to date from the late Pliocene (Bachmeyer & Pretzmann, 1971). Freshwater crabs are highly derived heterotremes and the more complete fossil record of heterotreme marine crabs indicates that earliest fossil heterotremes (Calappidae and Dorippidae) date back to the Early Cretaceous (144 mya). This indicates that the heterotremes underwent a post-Cretaceous radiation (Glaessner,

1969), which would mean that freshwater crabs may have first appeared at some time during the early to mid-Cenozoic era (65-30 mya). It is difficult to identify fossils of freshwater crabs because crabs found in fresh water could be species that live in fresh water but reproduce in sea water, such as is the case for the Chinese mitten crab *Eriocheir sinensis* (Grapsoidea: Varunidae).

1.2. History of freshwater crab taxonomy and systematics

All freshwater crabs were originally placed in a single family, the Thelphusidae (H. Milne Edwards, 1837), a name that was later changed to the Potamonidae (Ortmann, 1896) and then to the Potamidae (Table 1.1). In the late 19th and early 20th centuries, freshwater crab systematists still considered the world's freshwater crabs to belong to a single family and several distinct subfamilies were recognized (Rathbun, 1904, 1905, 1906). This was changed radically by Bott (1970), who revised freshwater crab higher classification to comprise three superfamilies containing 11 families (Table 1.1).

The classification schemes of Bott (1955, 1970) viewed the freshwater crabs as a polyphyletic assemblage, whereby each family - and even groups within each family - originated from a distinct group of marine crabs that each entered freshwater to give rise to the different families. This classification has since been challenged by a number of authors who carried out phylogenetic analyses of the freshwater crabs (Sternberg et al., 1999; Sternberg & Cumberlidge, 2001).

Many authors in the 20th century held the opinion that all freshwater crabs were related to the marine crabs in the superfamily Xanthoidea, although other superfamilies such as the Portunoidea were also suggested (see Bott, 1970).

Table 1.1 Recent classification schemes for the old world freshwater crabs

Bott, 1955	**Bott, 1970**	**Sternberg & Cumberlidge, 2001**
- Paleotropical Deckeniidae - Paleotropical Potamidae (Potamonidae)	**Parathelphusoidea (later Gecarcinucoidea Rathbun 1904)** - Gecarcinucidae - Parathelphusidae - Sundathelphusidae **Potamoidea (Ortmann 1896)** - Deckeniidae - Isolapotamidae - Potamidae - Potamonautidae - Sinopotamidae	- Deckeniidae - Gecarcinucidae - Parathelphusidae - Potamidae - Potamonautidae - Platythelphusidae
MARTIN & DAVIS, 2001	**KLAUS ET AL., 2006**	**CUMBERLIDGE ET AL., 2007 (in press)**
Potamoidea Ortmann, 1896 - Deckeniidae - Platythelphusidae - Potamidae - Potamonautidae **Gecarcinucoidea Rathbun, 1904** - Gecarcinucidae - Parathelphusidae	**Potamoidea Ortmann, 1896** - Potamidae - Potamonautidae **Gecarcinucoidea Rathbun, 1904** - Gecarcinucidae o Parathelphusinae o Gecarcinucinae - Deckeniidae o Deckeniinae o Globonautinae o Hydrothelphusinae	**Potamoidea Ortmann, 1896** - Potamidae - Potamonautidae o Potamonautinae o Hydrothelphusinae o Hydrothelphusinae ▪ Hydrothelphusini ▪ Deckenini ▪ Deckenina ▪ Globonautina **Gecarcinucoidea Rathbun, 1904** - Gecarcinucidae - Parathelphusidae

However, although well established, these ideas were based on only a few morphological characters, such as those of the male first pleopod (first gonopod G1) and the mandible. The first systematic attempt to discover the marine sister group of the freshwater crabs was that of Sternberg et al. (1999) who identified the Grapsoidea to be the most likely marine sister group of the freshwater crabs. These authors used a large morphological dataset, a wide taxonomic sample of most freshwater crab families, and multiple marine crab families as outgroups. Sternberg et al. (1999) concluded that the true freshwater crab families consisted of two distinct phylogenetic groups: the Trichodactylidae, a basal clade of the marine crab superfamily Portunoidea (Rodriguez, 1992) and a 'thelphusoid clade' (all other freshwater crab families), which was a subgroup of a larger clade that included the marine crab group Thoracotremata. These findings meant that all of the world's

families of freshwater crabs are not monophyletic *sensu lato*, but the monophyly of the neotropical Pseudothelphusidae and each of the paleotropical freshwater crab families was supported. Today, most authors recognize 7 or 8 families (Sternberg & Cumberlidge, 2001; Martin & Davis, 2001) (Table 1.1). Monophyly of the thelphusoid families argues against the polyphyletic hypotheses of Bott (1955, 1969, 1970, 1972) and Pretzmann (1973), which assumed ancestry from multiple marine crab lineages and multiple entries into freshwater for the freshwater crabs. According to Sternberg et al. (1999), during the Tertiary (beginning around 65 mya) populations of a widespread species of a heterotreme crab that was pre-adapted to freshwater migrated from shallow tropical littoral areas of the Tethys Sea into multiple freshwater environments of the adjacent tropical landmasses around the world (South America, Africa, and Eurasia; Sternberg et al., 1999).

A competing hypothesis for the origins of freshwater crabs is the 'archaic hypothesis' (also named the "vicariance" hypothesis) that considered the freshwater crabs to be a very ancient group that entered fresh water a single time (Rodriguez, 1986; Ng & Rodriguez 1995; Ng et al., 1995). Weaknesses to this hypothesis include the lack of fossil evidence for the assumed great age of the group, and the lack of cladistic support correlating phylogenies with the sequence of the breakup of the continents. Furthermore, Sternberg et al. (1999) argued that the archaic hypothesis rules out additional marine dispersal or even long distance land migrations because it assumes that the ancestral thelphusoid population on Gondwana had already acquired direct development and maternal care, was already restricted to the fresh water environment, and was incapable of crossing through sea water barriers.

1.3. Importance of freshwater crabs

Freshwater crabs are medically and economically important in some parts of the world. Some African freshwater crabs of the family Potamonautidae are linked to tropical diseases such as river blindness (onchocerciasis) and human lung fluke disease (paragonimiasis) (Cumberlidge, 1999) and freshwater crabs from Anhui and Fujian Provinces in China harbor the metacercariae of lung flukes (Dai et al., 1979). Human infestation by *Paragonimius* occurs following ingestion of raw or incompletely cooked freshwater crabs infected with metacerariae (Im et al., 1993). Large potamid and parathelphusid freshwater crabs are occasionally eaten by local populations in Asia (Ng, 1988) and large potamonautids are consumed in West Africa (Cumberlidge, 1999).

Freshwater crabs are still relatively poorly known and they have historically received less attention than their marine crab relatives (Sternberg & Cumberlidge, 2001). On the other hand, freshwater fish are easily the best known group of freshwater animals. The phylogenetic relationships of freshwater fish are better known than those of most large groups of invertebrates, and as a consequence freshwater fish are thought to be more significant models for zoogeography than are most freshwater invertebrates. Freshwater fish species that are exclusively found in fresh water and cannot cross saltwater barriers, yet occur on separate land masses provide a strong argument for a former pre-fragmentation continental origin. Interestingly, the controversy over the possible marine dispersal of freshwater cichlids from Africa and Madagascar to South America (Vences et al., 2001; Chakrabarty, 2004; Farias et al., 2000; Sparks, 2004) is similar to the discussion of the possible marine dispersal of freshwater crabs to

continental islands. Molecular clocks have a great potential to resolve some of these arguments, although researchers cannot agree on a verifiable method of molecular dating.

Invertebrates such as freshwater crabs, freshwater crayfish, freshwater mussels and several families of freshwater snails are just as intolerant of saltwater as species of exclusively freshwater fish. However, many of these freshwater invertebrates, unlike most fish, are also able to survive out of water provided there is a humid atmosphere, and some can cross narrow marine barriers being carried across either by birds or by drifting vegetation. The terrestrial ability of many species of freshwater crabs (including air-breathing and tolerance to dehydration) increases the potential for the possibility of the successful dispersal of these freshwater invertebrates across marine barriers.

1.4. Freshwater animals and biogeography

According to Banarescu (1990) freshwater animals can be divided into two major groups - primary aquatic freshwater animals of marine origin, and secondary aquatic freshwater animals of terrestrial origin, but the line between primary and secondary freshwater animals is blurred. In assessing whether a group is primary or secondary, historical biogeographers also take into consideration the salt tolerance of the relatives of a species: freshwater species with close relatives in the sea are typically considered secondary freshwater animals. Thus fish zoogeographers might reassign primary freshwater fish as secondary freshwater animals to allow for their possible movement through salt water. A third category includes amphibious animals that normally live in freshwater but are also found on land in places with a humid atmosphere, and can even disperse over short distances over land (Banarescu, 1990). Examples of this group

include some species of Old World freshwater crabs (e.g., Potamoidea, Gecarcinucoidea), land crabs (e.g., Grapsoidea) and crayfish (Astacoidea).

Monophyletic freshwater animal groups that have a circumglobal distribution and an origin that postdates continental fragmentation pose problems for zoogeographers because continental drift theory cannot be used to explain current distribution patterns.

In the absence of vicariance hypotheses, the ability of animals to disperse becomes important. Species can disperse by three methods (Pielou, 1979, cited in Banarescu, 1990):

1. Jump dispersal: the rapid movement of a number of individuals across a barrier followed by the successful establishment of a new colony. Jump dispersals are completed in a time frame that is much shorter than the life-span of an individual.
2. Diffusion: the gradual movement of populations across hospitable terrain for a period of many generations.
3. Secular migration: the very slow diffusion of populations over geological time that is often accompanied by drastic genetic modifications of the migrating animals, even by speciation.

Jump dispersal is the most appropriate of these methods when freshwater crabs are considered because it leaves several possibilities for dispersal routes across saltwater barriers (Banarescu, 1990). These include (1) direct movement in sea water across saltwater barriers, (2) short distance movement over humid earth resulting in radiations within landmasses (amphibious animals), and (3) occasional passive transportation across the sea (e.g., thiarid snails). Freshwater fish, large freshwater mussels, freshwater snails, and some crustaceans are strictly tied to freshwater and die when exposed to saltwater for

any length of time (Banarescu, 1990). The salinity tolerance of freshwater crabs has not been properly evaluated and so it is not clear whether they can move across sea water or even move short distances over land.

The distinction between primary and secondary freshwater animals was first proposed by Myers (1951) for freshwater fish and has been adopted by a number of ichthyologists. No equivalent classification is used by carcinologists and an attempt to do so by Banarescu (1990, Table A3) was criticized by Ng & Rodriguez (1995).

Secondary freshwater decapods could have occasionally crossed narrow sea water barriers, not necessarily directly through saltwater, but by rafting and other passive dispersal means. Freshwater crabs are known to be capable of dispersing short distances over land and of reaching one river basin from another. The question remains as to whether freshwater crabs are primary or secondary freshwater animals.

1.6. Ionic and osmotic regulation in freshwater crustaceans

It is of interest to see how members of predominantly marine groups have adapted to the challenges of life in freshwater, which contains only very minute amounts of ions. Beadle and Cragg (1940a, b, cited in Kinne, 1963) suggested that adaptation to life in freshwater by crustaceans proceeded in two main stages. The first stage is characterized by a reduction of the normal osmolality of hemolymph compared to that of marine crustaceans. The maintenance of a relatively high hemolymph osmotic pressure of freshwater crabs such as *Potamon fluviatile* (538 ± 97.5 mOsm/kg, Table 4.1.) in fresh water is associated with a large blood/tissue Cl^- gradient, and these animals can tolerate sudden increases in environmental salinity. The second stage is characterized by the

active reabsorption of salts from environmental fresh water against the concentration gradient, and a decrease in both hemolymph osmotic pressure and the blood/tissue Cl^- gradient. After animals have become adapted as described in stages one and two, exposure to higher salinities is stressful and eventually lethal. Shaw (1958b) found that freshwater animals could not survive for long in salinities with salt concentrations that were stronger than normal blood concentration. Another important adaptation of freshwater crabs to life in fresh water is a decreased permeability of the body surface to salts and water (Shaw, 1958b).

Once the transition from life in sea water to life in fresh water has been achieved, it is seldom reversed (Kinne, 1963). The ability to hyperosmoregulate in freshwater is often accompanied by the loss of the ability to adjust to either changing or increased salinities (Adolph, 1925, cited in Kinne, 1963). Freshwater crabs in fresh water use the gills as the primary organs of salt transport. Freshwater crabs can also live outside of water for short periods, despite compromising water and ion exchanges and ammonia excretion across the gills. Some freshwater crustaceans can also reclaim salt from the "urine" produced by their antennal organs as well as reclaiming salt across their gills. A partially amphibious life style divided between land and freshwater in which gills are often exposed to air presents the problem of maintaining a stable hemolymph concentration without the aid of the gills and compromises simple ammonia excretion.

Osmotic regulators maintain a constant internal osmotic concentration in the face of a constant osmotic gradient between body fluids and the external medium. In fresh water, active pumping of ions against a diffusion gradient is also essential to counteract

the efflux of ions. Osmotic conformers allow the internal osmotic concentration to follow that of the external medium (Randall et al., 2001; Pequeux, 1995).

Kinne (1963) classified different degrees of genetic adaptation to salinity and recognized four major groups separated by habitat – polystenohaline (saltwater), euryhaline (brackish water), oligohaline (freshwater) and holoeuryhaline (can move between different salinity regimes).

1. Polystenohaline animals are inhabitants of the ocean and have a relatively constant internal osmotic concentration that is equal to that of seawater. These animals are osmoconformers that practice ion and volume regulation but little or no osmoregulation.
2. Euryhaline animals are inhabitants of coastal, estuarine or brine habitats characterized by reduced, fluctuating, or extremely high salinities. Euryhaline animals are osmoregulators that have a reduced permeability of the carapace to water and salts, and well-developed mechanisms for the differential absorption and excretion of ions (e.g., *Carcinus maenas*, *Uca* sp., *Ocypode* sp.).
    a. hyperosmotic regulators are hyperosmotic in dilute seawater and more or less isosmotic in higher salinities (e.g., *Carcinus*).
    b. hyper-/hyposmotic regulators are hyperosmotic in dilute sea water and hyposmotic in higher salinities (e.g., semi-terrestrial and terrestrial crabs). Hyporegulation is always correlated with hyperregulation suggesting that hyper-hyporegulation is indicative of an advanced stage of genetic adaptation to either fluctuating or extreme salinities.

3. Oligohaline animals are inhabitants of freshwater. They possess well developed hyperosmotic regulation to meet the osmotic requirements of life in very dilute media, but their osmoregulatory mechanism collapses in salinities above 5 or 10 ppt.
4. Holoeuryhaline animals are inhabitants of sea water, brackish water and fresh water and are osmoregulators that can migrate from one medium to another, and can establish populations in a range of different salinities (e.g., *Eriocheir sinensis* or *Varuna litterata*).

Freshwater crabs are an oligohaline group because they live in fresh water and are not diadromous (Kinne, 1963). That author further differentiated between two groups of oligohaline freshwater animals, those that produce a urine that is more or less isosmotic to the hemolymph and those whose urine is hyposmotic to the hemolymph. For example, the European freshwater crab *Potamon fluviatile* (Schlieper & Hermann, 1930) and the African freshwater crab *Potamonautes niloticus* (Shaw, 1958a, b) both produce isosmotic urine and are relatively impermeable to salts. The marine-breeding Chinese mitten crab *Eriocheir sinensis* that lives in fresh water also produces isosmotic urine, but is relatively permeable to salts. *Eriocheir* can survive well in full-strength sea water (Shaw, 1958b), whereas *Potamon*'s ability for long term survival in sea water is unknown but assumed to be limited.

The second group of oligohaline animals that produce a urine that is hyposmotic to their hemolymph includes crayfish such as *Procambarus clarkii* and *Astacus* sp. whose urine is about 10% of their blood osmoconcentration. A 50 g crayfish in freshwater loses 600 mM of Cl^- daily (Prosser & Brown, 1961) and its hemolymph osmoconcentration is maintained within a regulated range. The urine output of crayfish decreases with

increasing salinity of the environmental water, and approaches zero when in a medium that is isosmotic to its blood (Scholles, 1933). Shaw (1958b) suggested that well-adapted freshwater animals typically produce copious, dilute hyposmotic urine and have a relatively low hemolymph osmotic concentration.

Freshwater crabs, like other aquatic animals that live in fresh water must spend energy to maintain their ionic and osmotic balance because they cannot completely isolate themselves from their environmental water due to their oxygen and nutrient requirements and their need to dispose of waste products (Randall et al., 2001). Fresh water typically has an osmolality of <50 mOsm/kg and the body fluids (hemolymph and interstitial fluids) of freshwater crabs in fresh water are always more concentrated than the environmental water. Freshwater crabs are therefore hyperosmotic to their fresh water environment and so have two kinds of osmoregulatory problems: they are in danger of their body fluids becoming too dilute due to the movement of water into their bodies down an osmotic gradient, and due to the continual loss of body salts to the surrounding water as ions move down a diffusion gradient (Randall et al., 2001). In freshwater crabs, the net gain of water and net loss of salts are reduced by adaptations such as a reduction in the permeability of the integument. Hemolymph volume is regulated by hormones from the endocrine system that control urine production. Animals in fresh water always face the potential long-term problem of gradually losing biologically important salts such as KCl, NaCl and $CaCl_2$, and must respond to this problem by the active transport of salts from the external dilute medium using Na^+/K^+ ion pumps on the gills and by the absorption of salts through their diet. Ions can move either passively through paracellular pathways, or actively across the epithelium/cell membrane through transcellular

pathways. Freshwater fish reabsorb electrolytes through their gills, while crayfish do the same through their gills and antennal glands (Randall et al., 2001) and land crabs (Gecarcinidae) minimize salt loss by a filtration-reabsorption system in the antennal glands analogous to the vertebrate kidney (Morris, 2002).

Not much is known about ionic regulation in freshwater crabs. In general there is an overall lowering of the hemolymph osmotic pressure in freshwater animals (Kirschner, 1991). This decreases the gradient between internal and external fluids, so reducing the osmotic influx/efflux of water, and reducing the energy demands needed to keep the internal concentration constant. For example in the true Old World freshwater crabs (e.g.*Potamon fluviatile, Potamonautes niloticus*, and *Potamonautes warreni*; Morris & van Aardt, 1998; Shaw, 1958b) sodium and chloride ion concentrations, blood ion compositions, and sodium loss indicated that these animals occupy an intermediate position between other marine crabs and freshwater crayfish (Tables 1.2, 1.3). Interestingly, these freshwater crab species also have a different sodium balance from the European crayfish *Austrapotamobius pallipes*.

Table 1.2 Comparison of the blood composition of a number of freshwater Crustacea (concentration in mmol/L) (Shaw, 1958b; Lutz, 1969; Onken & McNamara, 2002; Morris & van Aardt)

Species	Δ - NaCl	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	Author
Eriocheir sinensis	342	-	5.1	10	3.5	282	Scholles, 1933
Potamon fluviatile	340	337	8.5	18.1	-	300	Drilhorn & Portier, 1939
Potamonautes niloticus	271	259	8.4	12.7	-	242	Shaw, 1958b
Holthuisana. transversa	-	270.2	6.4	15.7	4.7	266.3	Greenaway & Mac Millen, 1978
Potamonautes warreni	-	238	4.5	13	4.69	271.5	Morris & van Aardt, 1998
Sudanonautes africanus	-	207.5	5.9	11.8	10.6	241.2	Lutz, 1969
Dilocarcinus pagei	-	190	9.7	10.2	2.8	206	Onken & McNamara, 2002
Astacus fluviatilis	228	152	3.1	12	2.5	176	Bogucki, 1934
Astacus fluviatilis	-	-	5.2	2.6	2.6	194	Scholles, 1933

Table 1.3 Rate of sodium loss rate in several decapod Crustaceans from different environments (Shaw, 1962)

Species	Habitat	Mean body wt (g)	Mean blood Na^+ concn mM/L	Na^+ loss rate (μM/hr)	Specific Na^+ loss rate (proportional) (μM/hr/50g)	Specific Na^+ loss rate (2/3's rule) (μM/hr/50g)	Reference
Carcinus maenas (from 40% SW)	BW	50	300	891	891	891	Shaw, 1961
Eriocheir sinensis (from 10% SW)	BW to FW	153	280 (estimated)	323	102	102	Shaw, 1961a
Potamonautes niloticus	FW	15	259	12	40	40	Shaw, 1958b
Austropotamobius pallipes	FW	12	186	1.8	7.5	7.5	Shaw, 1958c

At salinities from 0 to 20 ppt freshwater crabs maintain an internal osmolarity higher than the medium they are immersed in. Depending on external salinity levels, these crabs are either osmoregulators or osmoconformers. Most freshwater crabs osmoregulate over a range of external salinities before reaching a point when they cease to resist and switch to becoming osmoconformers.

Shaw (1958b) studied *P. niloticus* and determined sodium, potassium, calcium, chloride, and oxygen consumption, to test survival in different salinities. Crabs survived for more than 3 weeks in 25% and 50% SW, but when in 75% SW, survival was more variable (from 7 days to more than 3 weeks). In 100% SW, freshwater crabs showed a markedly different behavior: survival was low with some animals dying within a day or so, and with the remainder rarely surviving for longer than 4 days. The sample sizes used were small. Shaw (1958) concluded that survival of *Potamonautes* in high salinity water was similar to that for crayfish exposed to high salinity environments. Interestingly, the European freshwater crab (*Potamon*) survived for at least a month in full strength sea water, indicating that not all freshwater crabs are similarly intolerant of high saline environments.

Acclimating *P. niloticus* in steps, first to 50% SW, then to 75%, and then to full strength sea water had no beneficial effect on eventual survival times in full strength seawater. Shaw (1958b) indicated that longer acclimation steps over the critical concentration range (75-100% SW) did not extended survival times. Shaw (1958b) concluded that death in these animals resulted from an increase in the total osmotic concentration of the blood above a certain critical level. This was not surprising because this "behavior of the blood concentration with increasing sea water concentration is quite typical of the majority of true freshwater animals. Blood concentration slightly increases as the external concentration approaches the original blood concentration, but thereafter the rise in blood concentration is roughly proportional to the increase in the external concentration, the blood remaining slightly hyperosmotic" (Shaw, 1958b). Continued hyperosmosis in brackish water shows that the activity of ion uptake mechanisms is maintained in freshwater crabs. These ideas will be tested here in Asian freshwater crabs from two different families to establish whether the strategy of initial osmoregulation followed by a period of osmosconforming is a typical response.

Shaw (1958a, b) looked at the salinity tolerance of freshwater crabs in order to learn more about the physiological adaptations of these animals to fresh water. He thought that the initial adaptation to fresh water was a gradual reduction in the permeability of the body carapace to salts, and that the second adaptation was a reduction in the external salt concentration at which the saturation point of the active ion uptake mechanism was reached. The saturation point is reached because the uptake mechanism acquires a higher affinity for the ions being transported, which slows down the process (Shaw, 1958b).

Morris and van Aardt (1998) examined ion and water balance of the amphibious South African freshwater crab *Potamonautes warreni* from the point of view of pre-adaptations to life on land. Those authors found that osmotic pressure of the hemolymph was regulated (i.e., remained the same) when crabs were in 50% SW which increased after one week of exposure to 80% SW. *Potamonautes warreni* maintained hemolymph [Na^+] when in water concentrations up to and including 40% SW, but was unable to prevent increases in [Na^+] after two weeks exposure to 80% SW. The regulation of blood [Cl^-] failed (i.e., [Cl^-] increased) after one week in 80% SW, and [Mg^{2+}] increased after one week in 80% SW, but did not show an increase until the third week of exposure when held in 40% SW. Hemolymph [K^+] increased after two weeks in the group exposed to 80% SW only, but significant change was seen in hemolymph [Ca^{2+}] in any treatment.

In summary, *P. warreni* in high salinity sea water produced urine, that was isosmotic to its hemolymph, and reabsorbed 94% of the water and most of the ions in the primary filtrate. This water conservation mechanism is typical of other freshwater crabs placed in high salinity water: *Holthuisana transversa* reabsorbed 94% of the water, and *Potamon fluviatile* reabsorbed 54% (Greenaway, 1981; Harris, 1975; both cited in Morris & van Aardt, 1998). Lutz (1969) examined the salt and water balance of plasma, urine, and muscle of the amphibious West African freshwater crab *Sudanonautes aubryi* in response to desiccation. Even though 24% of the original body water was lost upon severe desiccation, there was no significant change in the levels of plasma chloride, calcium, and potassium, but there was a large increase in sodium concentration.

The tropical South American trichodactylid freshwater crabs belong to a separate phylogenetic lineage to the Old World freshwater crabs (Gecarcinucoidea, Potamoidea)

and as such make an ideal reference group, as do crayfish (Astacoidea), which are the temperate zone ecological equivalents of tropical freshwater crabs. Marine crabs found in fresh water such as *E. sinensis* are also of interest in this context (Banarescu, 1990).

The osmotic and ion-regulatory abilities of the hololimnetic trichodactylid freshwater crab *Dilocarcinus pagei* which is endemic to the Amazon and Paraguai/Parana river basins of South America was examined by Onken & McNamara (2002). This New World species as a representative of a phylogenetically separate lineage of New World freshwater crabs (Trichodactylidae) may not necessarily show the same adaptations as those seen in Old World freshwater crabs. The hemolymph osmolality of *D. pagei* is approximately 390 mOsm/kg (Onken & McNamara, 2002), which is about one-third sea water. This is a typical adaptation seen in species that are well adapted to life in fresh water, because a low hemolymph osmolality lowers the energy expenditure needed for salt uptake and reduces the outwardly-directed ionic gradient. Salt loss across the gills by diffusion and water entry by osmosis are both reduced by the low permeability of these animals to salt and water movements (Shaw, 1958b; Harris 1975; Greenaway, 1981; Morris & van Aardt, 1998).

Freshwater crabs do not have the ability to produce very dilute (hyposmotic) urine, and they cannot therefore help to reduce ion loss much with isosmotic urine, but they do lower the overall rate of urine production, as well as reabsorbing (rather than losing) the isosmotic fluid in the antennal gland. This strategy may be a water-conserving adaptation to an amphibious life (Greenaway, 1981; Harris, 1975; Morris & van Aardt, 1998). Hyperosmoregulating crabs compensate for ion loss by actively absorbing NaCl across their gill epithelia (Pequeux et al., 1988; Pequeux, 1995).

1.7 Project objectives

The objectives were to test the physiological tolerance to sea water of representatives of two Old World freshwater crab families (Parathelphusidae and Potamidae) in order to determine whether seawater is a barrier to their dispersal. To do this, the osmoregulatory ability of these animals was assessed by measuring their hemolymph osmolality after subjecting groups of male and female adult and juvenile crabs to a range of salinities. The results were interpreted in terms of the present day distribution patterns of these two families. The osmoregulatory ability and survival times of Asian freshwater crabs were compared to those of New World freshwater crabs (such as trichodactylids), marine-breeding land crabs (Grapsoidea), and freshwater crayfish (Astacoidea) in order to better understand potential evolutionary adaptations.

The questions asked were: How long can Asian freshwater crabs survive in sea water? Do freshwater crabs in different families respond differently? Do they osmoregulate or osmoconform? What is their isosmotic point? How does gender, age and water depth affect survival or osmoregulatory ability? How do freshwater crabs compare to crayfish and other crabs in fresh water? What are the biogeographic implications of these results?

MATERIAL AND METHODS

2.1. Animals

***Esanthelphusa dugasti* Rathbun, 1902 (Parathelphusidae).** Specimens were collected from a rice field in Chonburi Province, Thailand. The first group (n= 420) was collected on July 11, 2006 and the second group (n= 150) on July 30, 2006. Crabs were transported to the laboratory in rice bags with a small volume of water and sorted into aerated 1.2 or 1.5 L tanks in groups of 5 according to age (sub-adult or juvenile) and gender. Crabs were acclimated to laboratory conditions in freshwater at 30˚ ± 2˚C. A total of 418 sub-adults and juveniles (193 males and 225 females) survived the initial 3-day acclimation phase. The size range of the sub-adults was from cw 24.7 by cl 21.1mm, to cw 39.6 by cl 30.6 mm. The size range of juveniles was from cw 17.9 by cl 14 mm, to cw 28.1 by cl 26.5 mm. Sub-adult specimens had a notably thickened palm of the claw and this was used as an additional criterion to distinguish sub-adults from juveniles. No adult specimens of *E. dugasti* (cw > 40 mm) were present in the samples available. Specimens were identified by Pongrat Dumrongjatawana (Burapha University, Thailand) and Dr. Neil Cumberlidge (Northern Michigan University) using the identification keys in Bott (1970) and Chuensri (1974).

***Sayamia germaini* Rathbun, 1902 (Parathelphusidae).** Some 138 crabs weighing 20 kg were purchased from a local market in Nongkea, Saraburi Province on the 29th of July 2006, where they were on sale for human food consumption. Only adult crabs (cw > 40 mm) were available. Sixty adults (36M, 24F), measuring between cw 40.3 by cl 33.9 mm

and cw 63.3 by cl 53.6 mm survived the acclimation period. Crabs were transported back to the lab in rice bags in shallow water until they were put into individual aerated 1.2 or 1.5 L tanks and crabs acclimated to lab conditions in freshwater at 30˚ ± 2˚C.

Potamon smithianum **Kemp, 1923 (Potamidae, Potamiscinae, incertae sedis).** One hundred and twenty specimens were collected by hand on July 15th 2006 from Klung District, Chantaburi Province, Thailand from two different locations: during daylight at Klong Narai Waterfall (air temperature 28˚C, water temperature 25˚C), and at night in a waterfall stream on a fruit plantation in Ban Boi Vein Village (air temperature 26˚C, water temperature 24˚C). Crabs were transported to the lab in rice bags supplied with only a small volume of water and wet leaves and put into individual plastic tanks. The biggest adults (≥ cw 56.8 by cl 48.2 mm) were put into aerated 1.2 or 1.5 L tanks, smaller adults (≤ cw 27.9 by cl 22.8 mm) were put into non-aerated 0.4 L tanks, juveniles (from cw 17.7 by cl 15 mm to cw 34 by cl 33 mm) were put into non-aerated 0.125 L tanks. Newly-hatched crabs that had been dislodged from the mother's brood pouch, termed here 'mini' crabs, were too small to either handle, record their gender, or sample their hemolymph. These 'mini' juveniles (cw 4 by cl 4 mm to cw 18 by cl 18 mm) were put into non-aerated 0.25 L glass jars. Crabs were acclimated in freshwater at a temperature of 30˚ ± 2˚C, and 80 adults and juveniles (34M, 46F) and 20 mini juveniles survived long enough to include in the experiments.

Larnaudia chaiyaphumi **(Naiyanetr, 1982) (Potamidae, Potamiscinae).** One hundred and twenty specimens were collected from Chet Sa Noi Waterfall (air and water

temperature 27˚C) in Saraburi Province, Thailand, on the 29th of July 2006. Crabs were transported in rice bags with a small volume of water and put into individual plastic tanks, and acclimated in fresh water at 30˚ ± 2˚C. Adults and juveniles were put into non-aerated 0.4 L plastic tanks, while the "mini crabs" were put into non-aerated 0.25 L glass jars. Seventy-five adults and juveniles (43M, 32F) survived acclimation. The size range of the adults was from cw 31.3 by cl 25.3 mm to cw 47 by cl 39 mm. The juvenile size range was from cw 18.2 by cl 15 mm to cw 32 by cl 25.8 mm, and the 'mini' crabs measured from cw 11.2 by cl 8.8 mm to cw 20.4 by cl 19.1 mm.

2.2. Preparation of Media

The fresh water used was tap water that had been left standing for at least one day to eliminate added chlorine. Dilute sea water was prepared by adding fresh water to sea water drawn from the Burapha University sea water tank. Sea water concentration was determined using a salinometer and recorded in parts per thousand (ppt or ‰), which represents the physical quantity of kilograms of salt per kilogram of water in parts per thousand. The relationship between water osmolality (mOsm/kg) and salinity (ppt) is shown in Figure 2.1. Due to high air temperature (and high evaporation rate) in the lab, the salinity of the experimental media tended to increase even over short time periods, and so was therefore checked and adjusted daily.

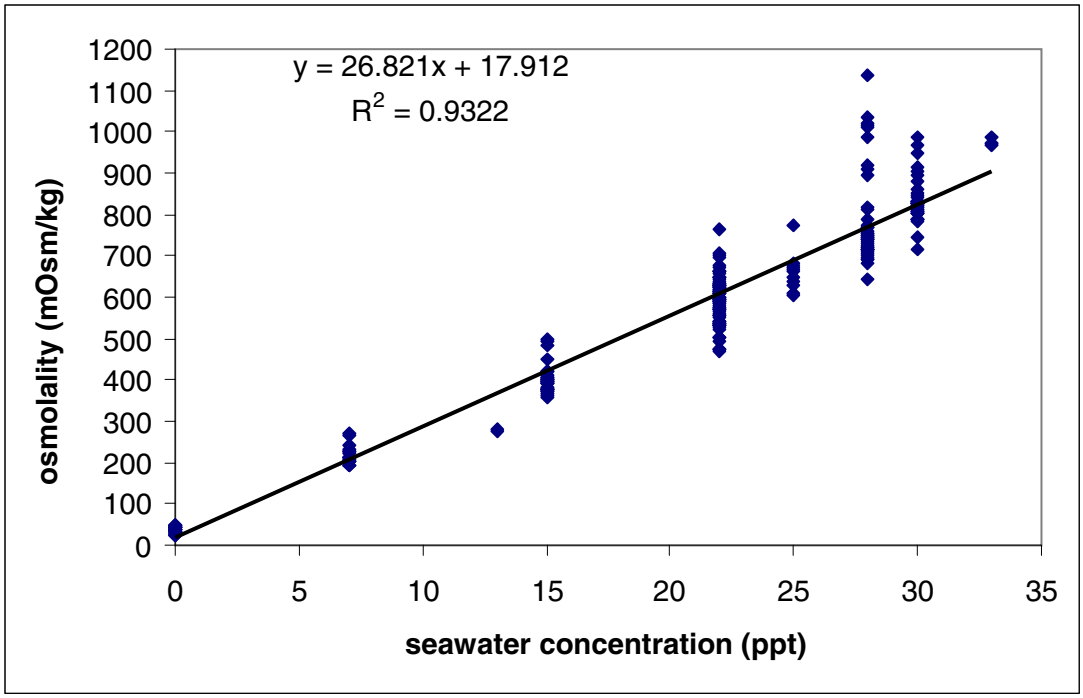


Fig. 2.1 Osmolality (mOsmol/kg) of prepared sea water concentration in parts per thousand (ppt)

2.3. Experimental groups

The initial acclimation phase allowed crabs time to recover from the stress of transportation and to acclimate to lab conditions. Following this, animals of both sexes and all age groups (adult, sub-adults, juveniles and newly hatched 'mini' juveniles) were divided into experimental groups and subjected to a series of different salinities. Experimental media were aerated in the 1.5 L and 1.2 L tanks, but not smaller tanks because they were too small to be both aerated and kept securely closed at the same time. The experimental media were maintained at 30˚ ± 2˚C and had salt concentrations ranging from 7 ppt (23.3% SW) to 33 ppt (110% SW). Control group animals were maintained in freshwater – 0.08 to 2 ppt. Crabs were subjected to a range of salinities in either shallow or deep water conditions. Animals subjected to shallow water were only partially immersed so that they could breathe air as well as water. This was achieved by

putting 250 mL of water in the 1.5 L tanks, 150 ml in the 1.2 L tanks, 59 ml in the 0.4 L tanks, 25 ml in the 0.125 L tanks, and less than 10 ml in the 0.25 L glass jar. Animals subjected to deep water were fully immersed and prevented from breathing air. The 1.5 L tanks contained 1200 mL, the 1.2 L tanks contained 900 mL, the 0.4 L tanks contained 225 mL, the 0.125 L tanks contained 100 mL water and the 0.25 mL glass jar contained no more than 20 mL water.

The first series of salinity experiments on *E. dugasti* and *P. smithianum* were performed over a period of 14 days, the second series on *E. dugasti* lasted for up to 11 days, while those on *L. chaiyaphumi* lasted for up to 12 days. The duration of the experiments was limited by the amount of time available at Burapha University, and by the number of crabs that could be bled each day for hemolymph osmolality measurements. Crabs were fed with tiny amounts of dried squid on days 6, 10, and 14 after the start of the experiment and water was changed every other day. It was aimed to have between 10 and 15 animals in each experimental group so that the results for *S. germaini, P. smithianum, L. chaiyaphumi,* and *E. dugasti* would be comparable, but the differences in the number of specimens for each species meant that not all experimental combinations were possible (Table 2.1).

2.4. Osmolality readings

Hemolymph osmolality was measured in mmol/kg on an automatic micro-osmometer (Wescor Vapro 5520) located in a room where the air temperature was 25˚C, because a stable temperature is needed to make accurate measurements. The osmometer uses a vapor pressure method (Wescor Handbook). A 10 µL sample of undiluted

hemolymph was pipetted onto a small, solute-free paper disc which was then inserted into a sealed sample chamber. After 90 seconds the display showed the reading of the solution in SI units (mmol/kg), which are equal to the commonly employed units of mOsm/kg (Wescor).

Table 2.1 Summary of all experimental groups and numbers sampled. *only sampled once before the start of the experiment.

water depth	**shallow water (salinity ‰)**									**deep water (salinity ‰)**			
Family / Species	fw	7	13	15	22	25	28	30	33	fw	22	25	30
PARATHELPHUSIDAE	Number sampled												
E.dugasti female sub adult	12	15	13	14	14	14	12	13	14	15			11
E.dugasti male sub adult	15	15	10	15	13			14		13			15
E.dugasti female juvenile	13		6		11			13			14		
E.dugasti male juvenile	13		4		15			15	5		20		17
S.germaini female adult	5										4		10
S.germaini male adult	5			10							5		10
POTAMIDAE													
P.smithianum female adult	10				12		13			7	2)	9	5
P.smithianum male adult	10				5		9			8	5	4	6
P.smithianum female juvenile	5				7								
P.smithianum male juvenile	5				8								
P.smithianum mini					20								
L.chaiyaphumi female adult	6*				6								
L.chaiyaphumi male adult	6*				6								
L.chaiyaphumi female juvenile	6				10								
L.chaiyaphumi male juvenile	6				12								
L.chaiyaphumi mini	10				10			10					

2.5. Osmotic regulation

The osmoregulation in crabs exposed to a range of salinities from fresh water to sea water was determined by sampling crab hemolymph (10-50µl) from each animal using a needle mounted on a syringe (1mL 25G 5/8 BD Latex Free Syringe with Precision Glide Needle 0.5mm x 16mm) inserted into the arthrodial membrane at the base of a posterior pereiopod. A 10µl hemolymph sample was read immediately. The hemolymph osmolality of sub-adult specimens of *E. dugasti* and adult *P. smithianum*,

was measured after 5 days, 9 days and 13 days, while that of juvenile *E. dugasti* and juvenile *P. smithianum* was measured only on day 13. In the second series of experiments, sub-adult *E. dugasti* hemolymph osmolality was measured after 5 days and 9 days, while that of juveniles was only measured after 9 days. Hemolymph osmolality of adult *S. germaini* and adult *L. chaiyaphumi* was measured after 5 days and 9 days while that of juvenile *L. chaiyaphumi* was measured after 8 and 12 days, and the 'mini' crabs were sampled after 12 days wherever possible.

2.6. Survival times in different salinities

The survival times of crabs exposed to different salinities was measured after transferring freshwater-acclimated crabs to their experimental media. The percent survival of a group of crabs in each salinity regime was recorded. Dead individuals were removed as soon as possible.

2.7. Analysis of osmolality data

Hemolymph osmolality (mmol/kg) was graphed for each salinity regime and for each day that the osmolality of the hemolymph was measured. The average osmolality of the experimental water was also plotted for comparison.

Repeated measures ANOVAs were performed on the datasets using SPSS Version 13.00 for Windows. This statistical test was chosen because the same measurement (hemolymph osmolality) was read several times for each specimen. The within-subjects (crabs) factor incorporated each set of repeated measures (e.g., days 5, 9 and 13), while the among-crab factor divided the crabs into groups (e.g., gender, age, water salinity, and

water depth). Groups with less than three osmolality readings were excluded due to limitations of the statistical test. Appendix A (for methods) provides the assumptions of this statistical test and the results of the assumption tests. The significance level for all tests was $p \leq 0.05$.

Esanthelphusa dugasti. Two GLM repeated measures ANOVA tests were used to analyze changes in hemolymph osmolality over time. The first test used data from days 5, 9 and 13, and only included sub-adults of both genders held in shallow water of different salinities. The second test used data from days 5 and 9, and included sub-adults of both genders held in both shallow and deep water conditions and a range of different salinities. Juveniles were only bled on the penultimate day of the experiment. A Univariate ANOVA was performed using osmolality data from day 13 of juvenile and sub-adult of *E. dugasti* of both genders held in shallow water of different salinities. Independent t-tests for water depth, age and gender on days 5 and 9 were also performed.

Sayamia germaini. A GLM repeated measures ANOVA test was performed using hemolymph osmolality data from days 5 and 9 of both male and female adult *S. germaini* subjected to different salinities in shallow and deep water.

Potamon smithianum. A GLM repeated measures ANOVA test using hemolymph osmolality of juvenile and adult that were bled after 5, 9 and 13 days in shallow water of different salinities was performed to analyze changes in hemolymph osmolality over time. A GLM Univariate ANOVA test was performed using hemolymph osmolality data taken on day 5 for juvenile and adult specimens of *P. smithianum* of different genders held in shallow and deep water of different salinities.

Larnaudia chaiyaphumi. Two GLM repeated measures ANOVA tests were run to analyze changes in hemolymph osmolality over time. The first test used data from adults held in shallow water at 22 ppt after 5 and 9 days, and a covariate held in 0 ppt. The second test used data juvenile and mini crabs held in shallow fresh water and in salinities of 22 ppt and 30 ppt without noting the gender of the specimens for 8 and 12 days. A GLM Univariate ANOVA test was performed using hemolymph osmolality data taken from day 12 for juvenile and "mini crabs" held in different salinities (0 ppt, 22 ppt and 30 ppt), and gender was not noted. Several independent t-tests were also performed to test for hemolymph osmolality differences due to age.

Species and family comparison. In order to compare the hemolymph of different species, the hemolymph osmolality results of groups of crabs of different gender, and age, were pooled for each salinity level and each day, and compared in one-way ANOVAs.

2.8. Analysis of survival data

Stepwise Forward Logistic Regression analysis was used to identify variables that were associated with survival of crabs in the different experimental media, because survival is a dichotomous dependent variable of interest (i.e., alive or dead; Menard, 2001). This analysis develops a model that predicts the probability that a subject will be classified into one or the other of the two categories of the dependent variable. The stepwise methods are used in exploratory analyses where theory development is of more concern than theory testing (Menard, 2001). A separate analysis for each species and each day (days 5, 9 and 13) was performed (Appendix A contains more background

information on logistic regression analysis). The dependent variable was survival (0 dead, 1 alive) and the independent variables included salinity, gender, age and water depth. Salinity was a continuous variable. Age, gender, and water depth were categorical variables and coded accordingly. Line graphs showing the percentage of animals that survived (in days) were plotted. Percent survival was chosen because total group numbers varied.

$$\text{Relative risk (RR) of dying} \quad \frac{\text{probability of dying when exposed to defined effect}}{\text{probability of dying of referent (control) group}}$$

was calculated within each species as well as between species for days 5, 9 and 14. A relative risk of 1 indicates no differences in risk between groups. A relative risk < 1 indicates that crabs survive better in the experimental group than in the control group. A relative risk > 1 indicates that crabs survive less well in the experimental group than in the control group (Rothman, 2002). When a relative risk is greater than 2 or smaller than 0.5, a finding of increased or decreased relative risk can be accepted (Science, Technology and Law Panel, 2002).

RESULTS

3.1 *Esanthelphusa dugasti*

3.1.1 *Hemolymph osmolality in deep and shallow water at different salinities*

Hemolymph osmolality in all control groups held in shallow fresh water varied among individuals (313 to 450 mOsm/kg) but was always much higher than the osmolality of fresh water in which they were held (30 to 85 mOsm/kg), indicating that *E. dugasti* maintains its internal environment hyperosmotic to its external environment (Fig. 3.1a). Similarly, when *E. dugasti* was subjected to shallow water with a salinity of 7 ppt, the osmolality of the water (200 to 272 mOsm/kg) was less than that of the hemolymph (313 to 500 mOsm/kg) (Fig. 3.1b). When *E. dugasti* was subjected to shallow water at 13 ppt (275 to 410 mOsm/kg) mean hemolymph osmolality was maintained above this range (i.e., hyperosmotic). The hemolymph of females bled after 5 days in 13 ppt water was isosmotic, while that of males bled after 9 days in 13 ppt diluted sea water was hyperosmotic (Fig. 3.2a). When *E. dugasti* was subjected to shallow water at 15 ppt (370 to 490 mOsm/kg) their mean hemolymph was hyperosmotic. After 13 days in 15 ppt, hemolymph osmolality range of male sub-adults of *E. dugasti* was more variable and values above as well as below the osmoality of the 15 ppt sea water (480 mOsm/kg) were recorded.

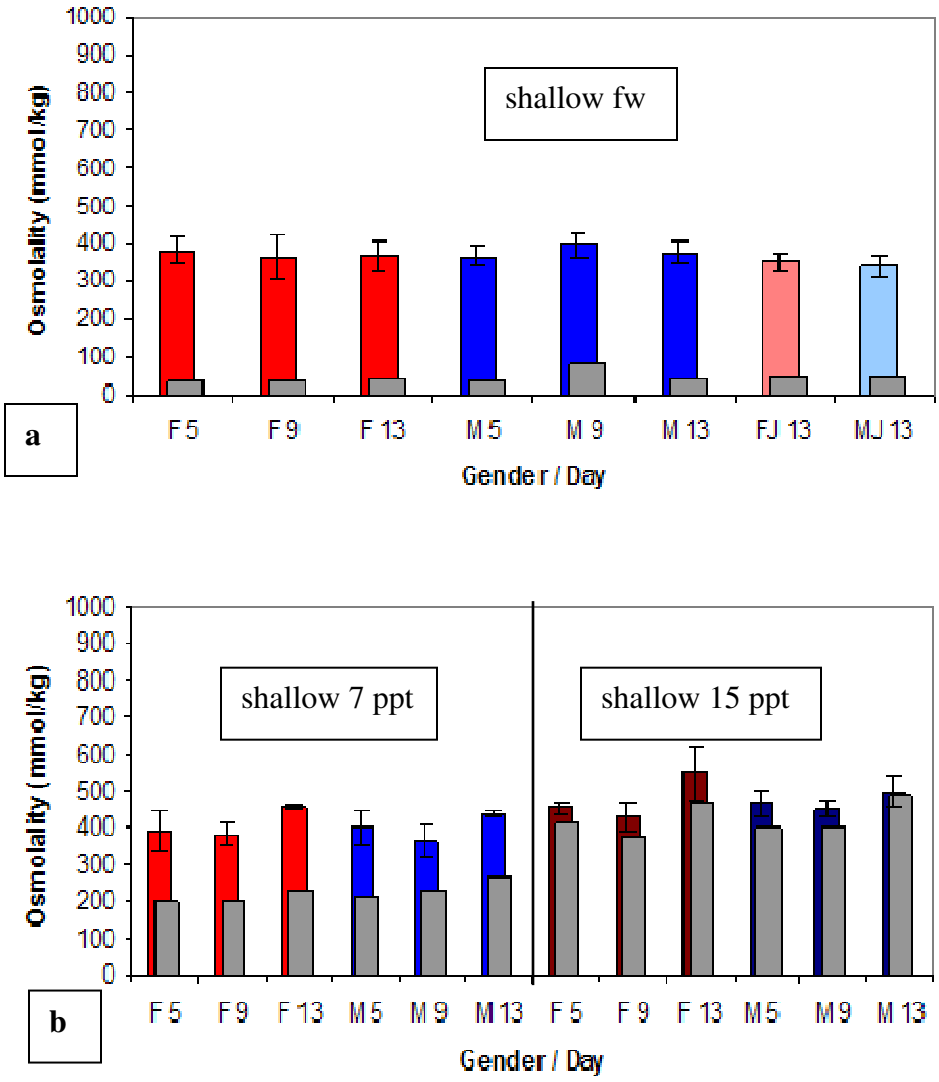


Fig. 3.1 Hemolymph osmolality of *Esanthelphusa dugasti* (Parathelphusidae) held in shallow water at 0, 7 and 15 ppt. Colored bars represent hemolymph osmolality, error bars = standard deviation, grey bars represent water osmolality. The x-axis shows the number of days that crabs were subjected to a particular salinity. F = female, M = male, J = juvenile, number = # of days into experiment. a) crabs held in shallow fresh water. Red = ♀ sub-adults, n = 12, 7, 5, respectively, blue = ♂ sub-adults n = 12, 10, 9, respectively, pink = ♀ juvenile n = 12; and light blue = ♂ juvenile, n = 8. b) crabs held in shallow sea water of 7 and 15 ppt. Red = ♀ adult n = 13, 6, 2, respectively, blue = ♂ sub-adult n = 13, 4, 2, respectively in 7 ppt sea water, dark red = ♀ sub-adult n = 12, 9, 4, respectively; and dark blue = ♂ sub-adult , n = 13, 8, 2, respectively in 15 ppt sea water.

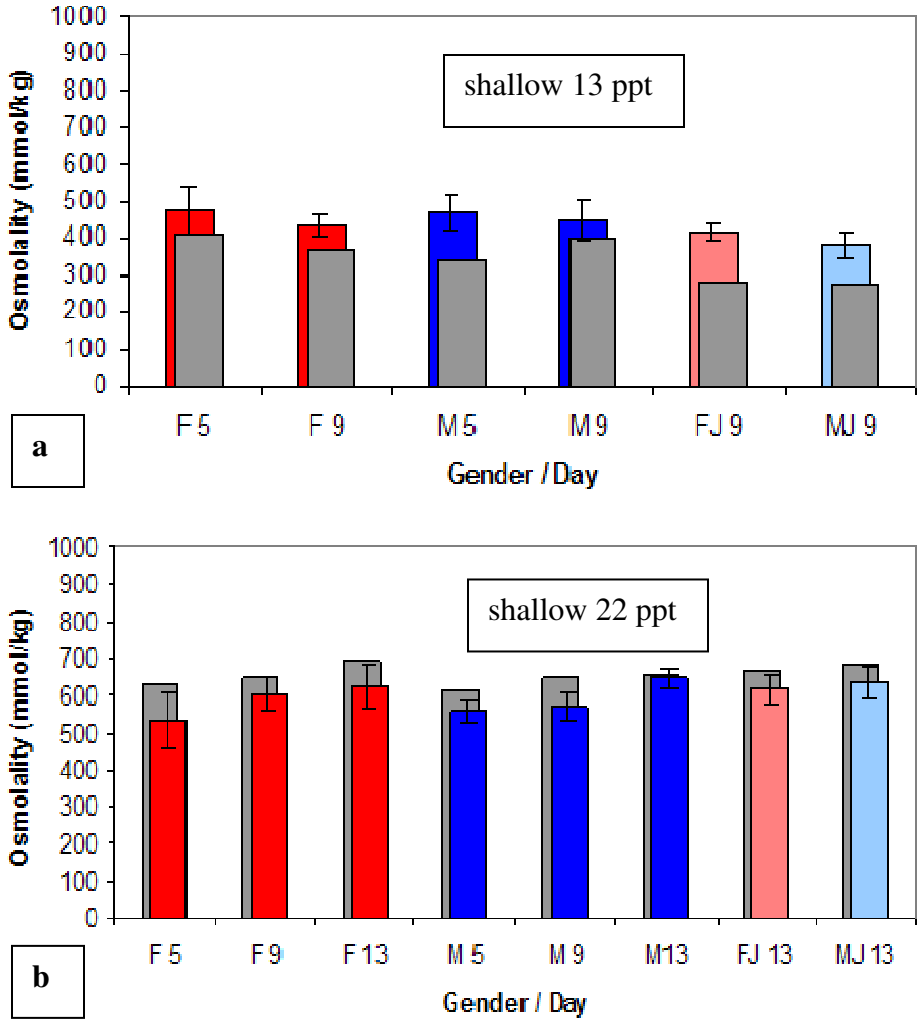


Fig. 3.2 Hemolymph osmolality of *Esanthelphusa dugasti* (Parathelphusidae) held in shallow water at 13 and 22 ppt. Colored bars represent hemolymph osmolality, error bars = standard deviation, grey bars represent water osmolality. The x-axis shows the number of days that crabs were subjected to a particular salinity. F = female, M = male, J = juvenile, number = # of days into experiment. a) crabs held in shallow 13 ppt sea water. Red = ♀ sub-adult n = 10, 7, respectively, blue = ♂ sub-adult n = 9, 7, respectively, pink = ♀ juvenile n = 6; light blue = ♂ sub-adult n = 4. b) crabs held in shallow 22 ppt sea water. Red = ♀ sub-adult n = 12, 4, 3, respectively, blue = ♂sub-adult n = 8, 5, 3, respectively, pink = ♀ juvenile n = 10; and light blue = ♂ juvenile n = 12.

The mean hemolymph osmolality of *E. dugasti* in shallow water at 22 ppt was 534 to 636 mOsm/kg which is below that of the holding water (631 to 690 mOsm/kg). In some groups of crabs the range of hemolymph osmolality was isosmotic to the holding water. For example after 13 days in 22 ppt, male juveniles and one male sub-adult had hemolymph osmolalities greater than the water osmolality (Fig. 3.2). The mean hemolymph osmolality in *E. dugasti* in shallow sea water (28, 30 and 33 ppt) was less than that of the surrounding water. However, the osmolality of water in the experimental tanks varied especially at 30 ppt (830 to 986 mOsm/kg). Male sub-adults subjected to 30 ppt water had a mean hemolymph osmolality that increased over time as the water osmolality increased (Fig. 3.3). In deep fresh water (53 mOsm/kg) the hemolymph osmolality of *E. dugasti* was between 328 and 453 mOsm/kg (hyperosmotic, Fig. 3.4) similar to that of crabs in shallow fresh water. Male and female juvenile *E. dugasti* that had been held for 9 days in tanks containing deep sea water at 22 ppt had an hemolymph osmolality that was essentially isosmotic to the water (480 mOsm/kg). In deep and shallow full strength sea water (30 ppt, 823 to 858 mOsm/kg) the hemolymph osmolality of crabs was hyposmotic (Figs. 3.4b, 3.3b).

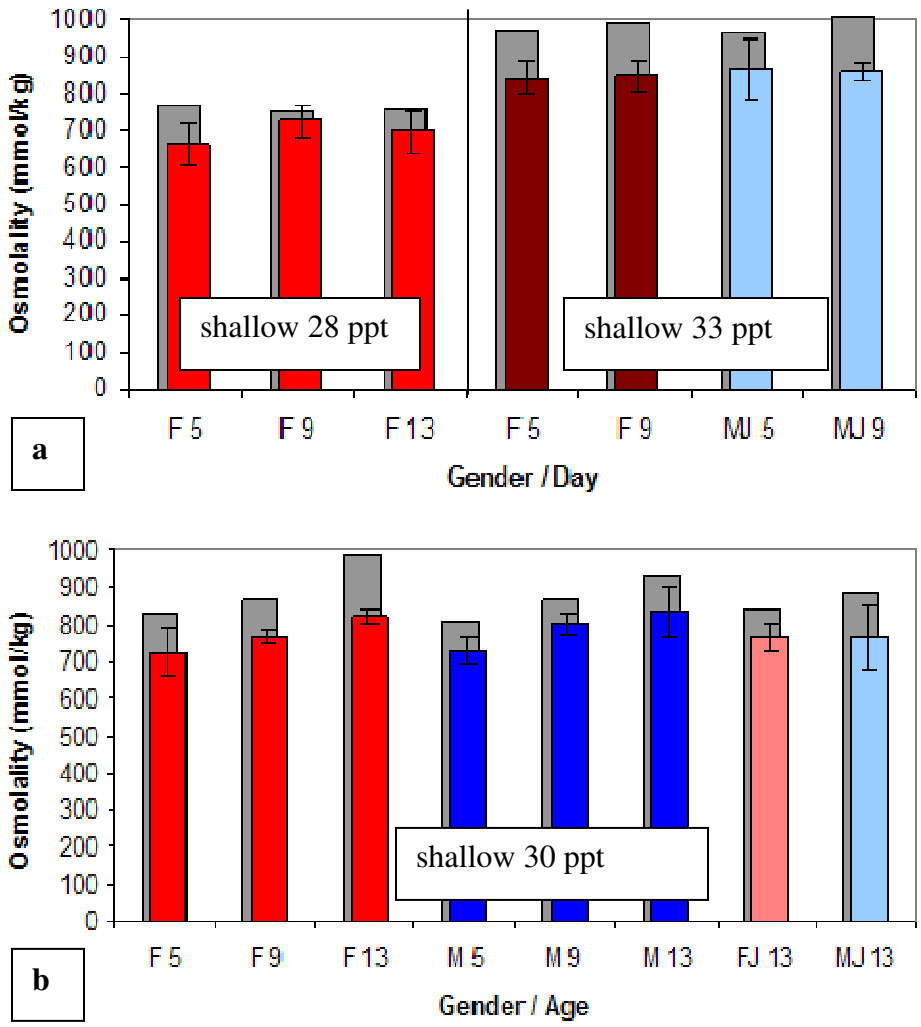


Fig. 3.3 Hemolymph osmolality of *Esanthelphusa dugasti* (Parathelphusidae) held in deep 0, 22 and 30 ppt water. Colored columns represent hemolymph osmolality, error bars = standard deviation. The grey columns represent water osmolality. The x-axis shows the number of days that crabs were subjected to a particular salinity. F = female, M = male, J = juvenile, number = # of days into experiment. a) crabs held in shallow 28 and 33 ppt sea water. Red = ♀ sub-adult n = 11, 8, 4, respectively, in 28 ppt sea water, brown = ♀ sub-adult n = 6, 2, respectively, light blue = ♂ juvenile n = 4, 2, respectively, in 33 ppt shallow water. b) crabs held in shallow 30 ppt sea water. Red = ♀ sub-adult n = 13, 3, 3, respectively, blue = ♂ sub-adult n = 13, 4, 4, respectively, pink = ♀ juvenile n = 9; light blue = ♂ juvenile n = 10.

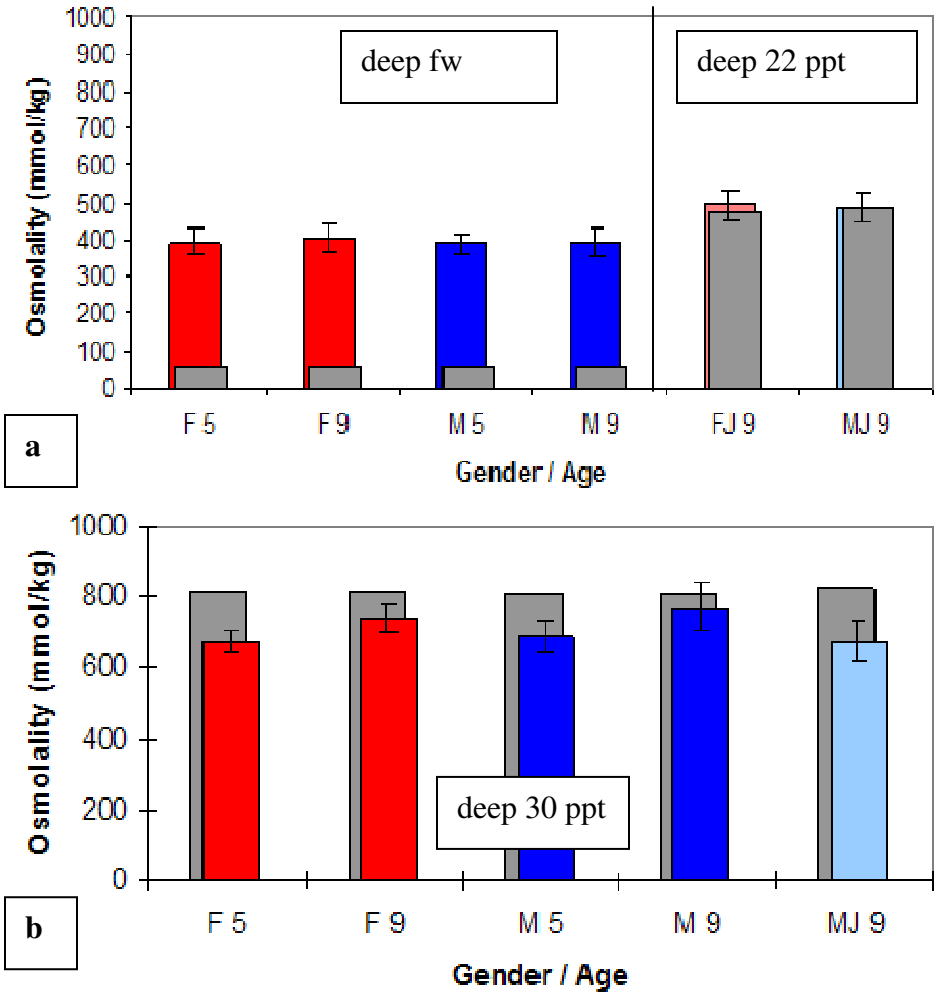


Fig. 3.4 Hemolymph osmolality of *Esanthelphusa dugasti* (Parathelphusidae) held in deep fresh water and at 22 and 30 ppt. Colored columns represent hemolymph osmolality, error bars = standard deviation. The grey columns represent water osmolality. The x-axis shows the number of days that crabs were subjected to a particular salinity. F = female, M = male, J = juvenile, number = # of days into experiment. a) crabs held in deep fresh water and in deep 22 ppt sea water. Red = ♀ adult n = 15, 14, ♂ respectively, blue = ♂adult n = 12, 10 respectively in fresh water, pink = ♀ juvenile n = 10, light blue = ♂ juvenile n = 15 in 22 ppt deep sea water. b) crabs held in 30 ppt deep sea water. Red = ♀ adult n = 10, 3, respectively, blue = ♂ adult n = 12, 5, respectively; light blue = ♂ juvenile n = 12.

3.1.2 *Factors affecting hemolymph osmolality in shallow and deep water at different salinities*

Shallow water

The hemolymph osmolality of sub-adult male and female specimens of *E. dugasti* held in shallow water of different salinities (ranging from 0 to 30 ppt) for 5, 9 and 13 days was significantly affected by the length of exposure (in days) to a particular salinity and tended to increase over time (GLM-repeated measure ANOVA, p = 0.00, Table B1, B2). The mean hemolymph osmolalities between male and female groups were found to be significantly different (p = 0.00, Table B3). In general the hemolymph osmolality of crabs subjected to shallow water was significantly different between groups (p = 0.00), as can be seen in Table 3.1 (Table B4). Average hemolymph osmolalities in crabs of each salinity group were significantly different except in crabs held in 0 and 7 ppt.

Table 3.1 Homogenous subsets of post-hoc Tukey tests of repeated measures ANOVA using osmolality data of *E. dugasti* of both genders subjected to different salinities for 5, 9, and 13 days. Mean mOsm/kg for homogeneous groups are displayed. Based on Type I sum of squares. The error term is mean square (Error) = 712.441. Uses harmonic mean sample size = 5.697.

	salinity	***N***	***subset***				
	ppt		**1**	**2**	**3**	**4**	**5**
Tukey HSD	0	13	382.71				
	7	4	397.75				
	15	6		476.44			
	22	6			588.27		
	28	4				709.5	
	30	7					770.42
	Sig.		0.92 NS	1	1	1	1

Effect of water depth

Hemolymph osmolality of sub-adult male and female *E. dugasti* that had been exposed to deep and shallow water at different salinities (ranging from 0 to 33 ppt) for 5 and 9 days was significantly affected by the length of exposure (in days) to a particular

salinity and tended to increase over time (GLM-repeated measures ANOVA, p = 0.00; Table B5, B6). Hemolymph osmolalities between males and females (p = 0.01) held at different salinities (p = 0.00) were significantly different between groups (Table B7). Hemolymph osmolality of the majority of the experimental groups was significantly different (p = 0.00) except for three pairs of combinations: 0 and 7 ppt, 13 and 15 ppt and 28 and 30 ppt, which are found in the same subset (Tables 3.2, B8).

Table 3.2 Homogenous subsets of the post-hoc Tukey tests of repeated measures ANOVA using osmolality data of *E. dugasti* sub-adults of both genders in deep and shallow water depths for 5 and 9 days. Means mOsm/kg for homogeneous groups are displayed. Based on Type I sum of squares. The error term is mean square (Error) = 980.404. Uses harmonic mean sample size = 8.511.

	salinity	**N**	**Subset**					
	ppt		**1**	**2**	**3**	**4**	**5**	**6**
Tukey HSD(	7	10	374					
	0	41	391.09					
	15	17		446.74				
	13	14		457.64				
	22	9			571.78			
	25	4				634.38		
	28	8					695.69	
	30	15					731.5	
	33	4						850
	Sig.		0.97	1	1	1	0.32 NS	1

Effect of age

The hemolymph osmolality of *E. dugasti* of different ages and both genders held in shallow water for 13 days was significantly influenced by both age (p = 0.00) and salinity (p = 0.00) (GLM-Univariate ANOVA, Table A9), except for two pairs of groups (7 and 15 ppt, 22 and 28 ppt, Table A10), and five homogenous subsets were found for the six different salinities (Table 3.3).

Table 3.3 Homogenous subsets of the Univariate ANOVA using osmolality data from *E. dugasti* of different ages and gender in shallow water after 13 days. Means mOsm/kg for homogeneous groups are displayed. Based on Type I sum of squares. The error term is mean square (Error) = 2108.863. The group sizes are unequal. The harmonic mean of the group sizes, 7.790, is used. Type I error levels are not guaranteed.

	salinity	***N***	***Subset***				
Tukey HSD	**ppt**		**1**	**2**	**3**	**4**	**5**
	0	34	358.62				
	7	4		447.25			
	15	6			531.17		
	22	28				629.89	
	28	4				697.25	
	30	26					783.88
	Sig.		1	1	1	0.05	1

Effects of water depth after 5 and 9 days

Independent t-tests indicate that the hemolymph osmolality was significantly lower for male sub-adults held for 5 days held in deep fresh water than when held for 5 days in shallow fresh water. In high salinities, hemolymph osmolality was significantly lower for male and female sub-adults held for 5 days in deep, compared to levels of groups in shallow 30 ppt water and was also significantly lower for male and female sub-adults held for 9 days in deep, compared to levels in groups in shallow 22 ppt water (Table 3.4).

Table 3.4 Summary of independent samples t-tests that showed significant differences in hemolymph osmolality between sub-adult *E. dugasti* held in shallow vs deep water. Equal variances were assumed based on F test.

		t-test for Equality of Means						
deep vs shallow	**day**	**t**	**df**	**Sig. (2-tailed)**	**Mean Difference**	**Std. Error Difference**	**95% CI of the Difference**	
							Lower	Upper
0 ppt M	Day 5	-2.28	22	0.03*	-25.08	10.99	-47.87	-2.3
22 ppt F	Day 9	5.24	12	0.0002*	130.65	24.9	76.38	184.91
22 ppt M	Day 9	4.63	18	0.0002*	90.67	19.59	49.52	131.82
30 ppt F	Day 5	2.53	21	0.02*	55.92	22.13	9.9	101.93
30 ppt M	Day 5	2.64	23	0.01*	44.06	16.67	9.59	78.54

Effects of age in full strength sea water

Independent t-tests indicate that the hemolymph osmolality was significantly higher ($p = 0.04$) in female sub-adults than in juveniles in 30 ppt shallow water for 13 days, and significantly higher ($p = 0.01$) in male sub-adults than in juveniles when subjected to 30 ppt deep water (Table B11).

3.1.3. *Survival in different salinities*

Effects of salinity and water depth on survival

Binary forward stepwise logistic regression on day 5 found that both the salinity regime and the water depth had a significant effect on survival (change in -2LL if term removed from model: -2LL of salinity = 4.890, df = 1, $p = 0.027$ and -2LL of water depth = 6.519, df = 1, $p = 0.011$). The variables gender, age, interaction of age or gender with salinity, and interaction of water depth with salinity were not significant to be in the reduced model. The overall correct classification of the reduced model was 85.6%. However, only a small amount of the variation associated with the log odds of being alive after 5 days could be explained by the reduced model (Nagelkerke $R^2 = 0.048$, -2LL = 304.069; Table 3.5).

Table 3.5 Variables in reduced logistic regression equation of survival data of *E. dugasti* after 5 days

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1	water depth (shallow)	-.882	.401	4.830	1	.028 **	.414
	constant	2.474	.368	45.178	1	.000 **	11.875
Step 2	salinity	-.030	.014	4.524	1	.033 **	.971
	water depth (shallow)	-.942	.404	5.421	1	.020 **	.390
	constant	3.095	.485	40.767	1	.000 **	22.087

Effects of salinity and age on survival

Age and salinity was found to have a significant effect on survival after 9 days (change in -2LL if term removed from model: -2LL of age = 46.215, df = 1, p = 0.00 and -2LL of salinity = 46.215, df = 1, p = 0.00). Variables such as gender, water depth, the interaction of age or gender with salinity, and the interaction of salinity or gender with water depth were not significant in the full model logistic regression equation. The overall correct percentage of classification of the reduced model is 68% and only a small variation in the log odds of being alive after 9 days can be explained by the reduced model (Nagelkerke R^2 = 0.196, -2LL = 451.104, Table 3.6).

Table 3.6 Variables in reduced logistic regression equation of survival data of *E. dugasti* after 9 days

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1	age (adult)	-1.334	.250	28.416	1	.000 *	.263
	constant	1.358	.216	39.615	1	.000 *	3.889
Step 2	salinity	-.054	.011	25.125	1	.000 *	.947
	age (adult)	-1.693	.270	39.251	1	.000 *	.184
	constant	2.602	.343	57.648	1	.000 *	13.497

Both age and salinity regime also had a significant effect on survival model after 13 days (change in -2LL if term removed from model: -2LL of salinity = 35.628, df = 1, p = 0.00 and -2LL of age = 29.699, df = 1, p = 0.00). Variables such as gender, water depth, the interaction of age or gender with salinity, and the interaction of gender or salinity with water depth were not significant and were not in the reduced model. The overall correct classification of the reduced model is 65.1% and only a small amount of the variation in the log odds of being alive after 13 days could be explained by the reduced model (Nagelkerke R^2 = 0.170, -2LL = 473.555; Table 3.7).

Table 3.7 Variables in reduced logistic regression equation of survival data of *E. dugasti* after 13 days

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1	salinity	-.043	.009	21.303	1	.000 *	.958
	Constant	.598	.198	9.154	1	.002 *	1.818
Step 2	salinity	-.059	.011	31.477	1	.000 *	.943
	age adult	-1.293	.248	27.248	1	.000 *	.274
	Constant	1.720	.307	31.355	1	.000 *	5.585

3.1.4 *Percent survival*

Survival was calculated as percent survival over time for each experimental group (see Figs. 3.5 to 3.7). Table 3.8 shows the number of animals of each experimental group at the beginning of the experiment.

Table 3.8 Experimental groups of *E. dugasti* and their sample sizes at the start of the experiment (F = female, M = male, A = sub-adult, J = juvenile). All groups were in shallow water, except for those marked #

Group	**N**	**Group**	**N**	**Group**	**N**	**Group**	**N**
FA control	12	MA 15 ppt	5	FJ control	13	control deep FA #	15
MA control	15	FA 22 ppt	14	MJ control	13	control deep MA #	13
FA 7 ppt	15	MA 22 ppt	13	FJ 22 ppt	11	FJ 22 ppt deep #	14
MA 7 ppt	14	FA 25 ppt	14	MJ 22 ppt	15	MJ 22 ppt deep #	20
FA 13 ppt	13	FA 28 ppt	12	FJ 30 ppt	13	FA 30 ppt deep #	11
MA 13 ppt	10	FA 30 ppt	13	MJ 30 ppt	15	MA 30 ppt deep #	15
FA 15 ppt	14	MA 30 ppt	14	MJ 33 ppt	5	MJ 30 ppt deep #	17

The highest percent survival was found in the control group (0.8 to 2 ppt), compared to those held in 7 or 13 ppt (Fig. 3.5). In 7 ppt, survival after 14 days was the same for both female and male sub-adults (Fig. 3.5b). While in 13 ppt male sub-adults survived slightly better than female sub-adults (Fig. 3.5c). In general, survival decreased over time in all the salinities, but there was no difference between the decline in survival of *E. dugasti* subjected to increased salinities (15, 22, 25, 28 or 30 ppt) for 14 days (Figs. 3.6a-d). For most groups the relative risk (RR) of dying after 14 days was small (<2). Increased RR was associated with males in shallow water at 7, 15 and 22 ppt (RR = 2.17,

R = 2.17, and R = 2.12, respectively; Table B15). Juveniles subjected to 22 or 30 ppt showed a higher percentage of survival than adults subjected to the same salinity. Decreased RR of dying after 14 days was associated with male (RR = 0.32) and female juveniles (RR = 0.23) in 22 ppt after 14 days compared to sub-adults and in male juveniles (RR = 0.19) in 30 ppt after 14 days compared to sub-adults (Table B16). No male juveniles of *E. dugasti* survived for longer than 12 days in 33 ppt seawater, and only 40% of female sub-adults survived in 33 ppt seawater survived for 9 days (Fig. 3.6e).

Sub-adult crabs in shallow and deep fresh water survived similarly well over a 9 day period (Figs. 3.5a, 3.7a). Juveniles of both genders survived for at least 11 days in deep water at 22 ppt (Fig. 3.7b). Sub-adults of both genders and male juveniles also survived for at least 11 days in deep 30 ppt sea water.

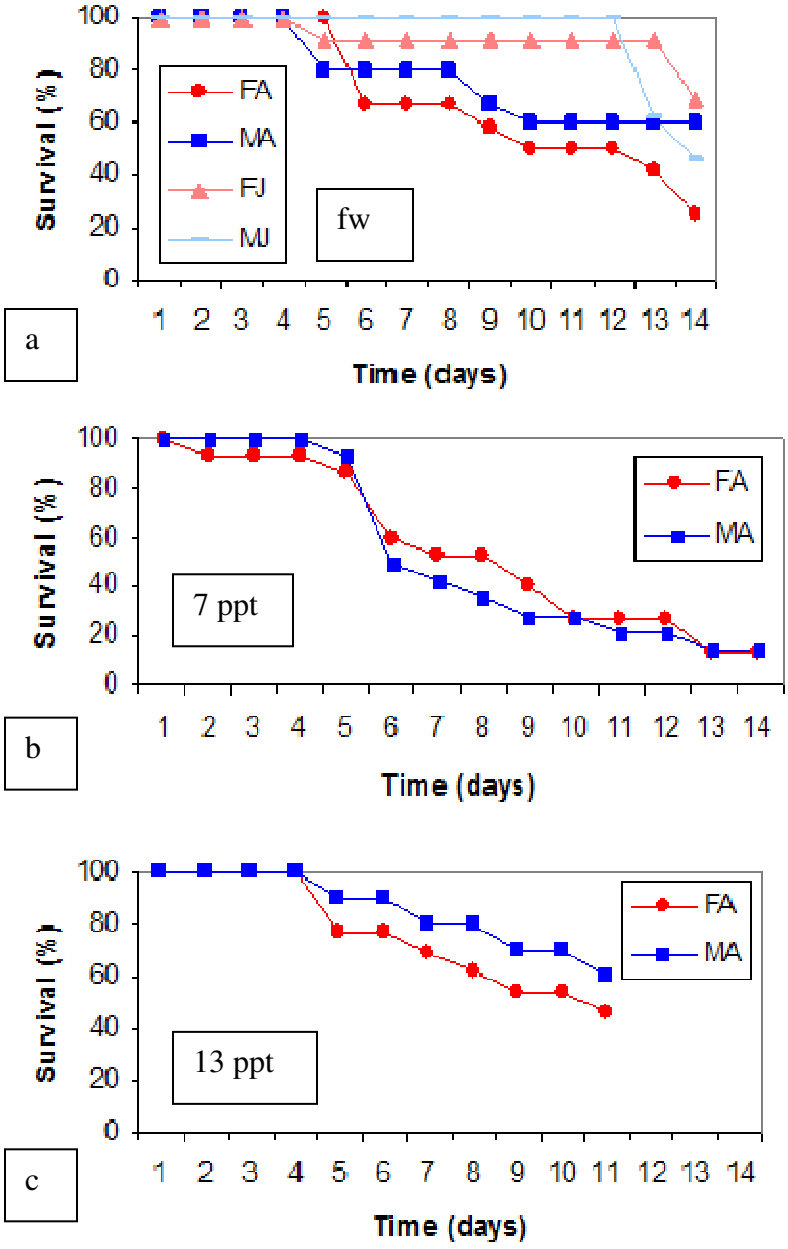
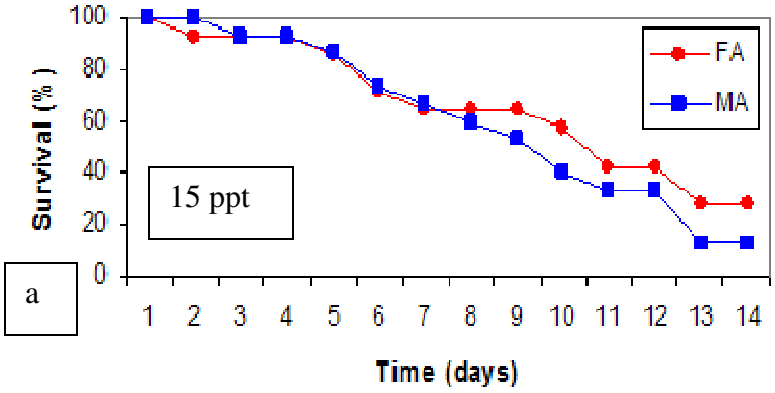
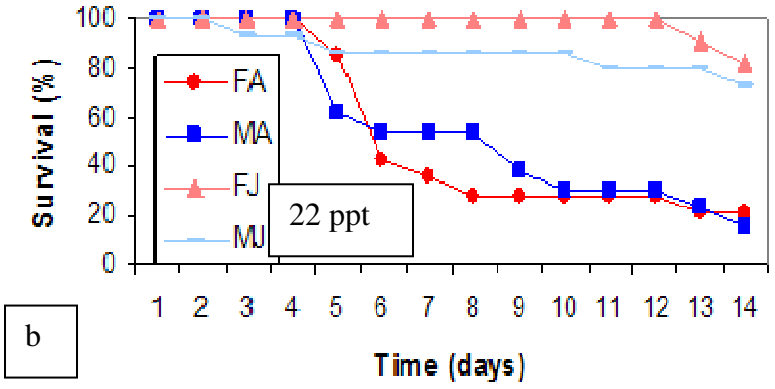


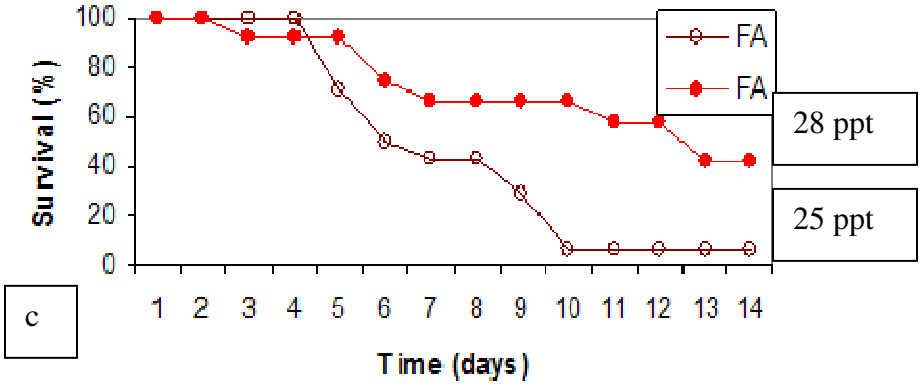
Fig. 3.5 Percent survival of *E. dugasti* in shallow water at 3 different salinities (a 14 days in 0 ppt, b 14 days in 7 ppt and c 11 days in 13ppt).


Survival (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (days)
FA
MA
15 ppt

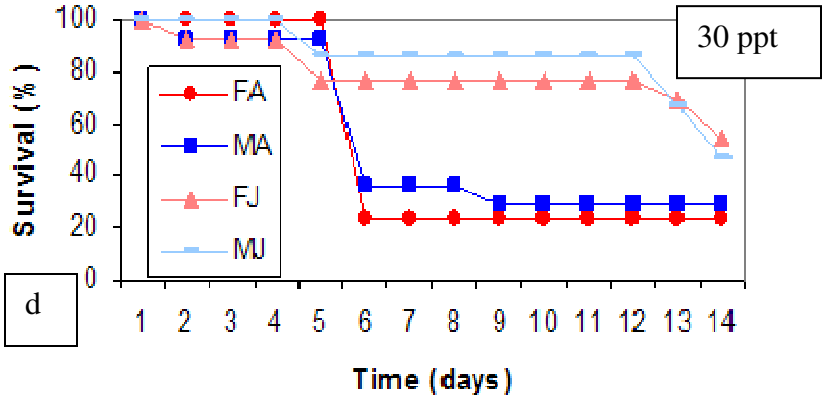
a


Survival (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (days)
FA
MA
FJ
MJ
22 ppt

b


Survival (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (days)
FA
FA
28 ppt
25 ppt

c


Survival (%)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (days)
FA
MA
FJ
MJ
30 ppt

d

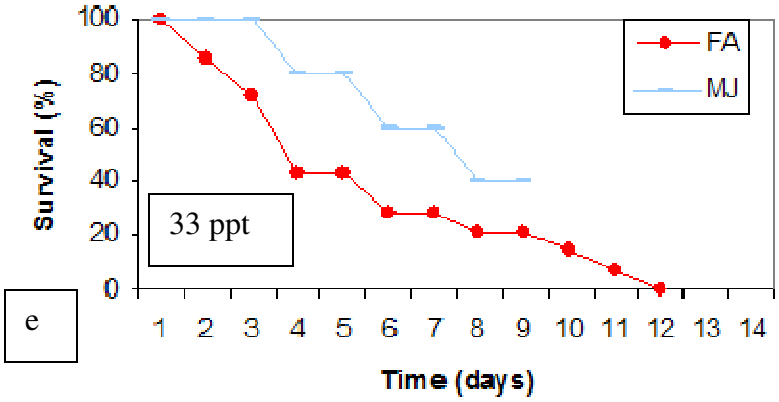


Fig. 3.6 Percent survival of *E. dugasti* over up to 14 days in shallow water at 6 different salinities (15, 22, 25, 28, 30 and 33 ppt).

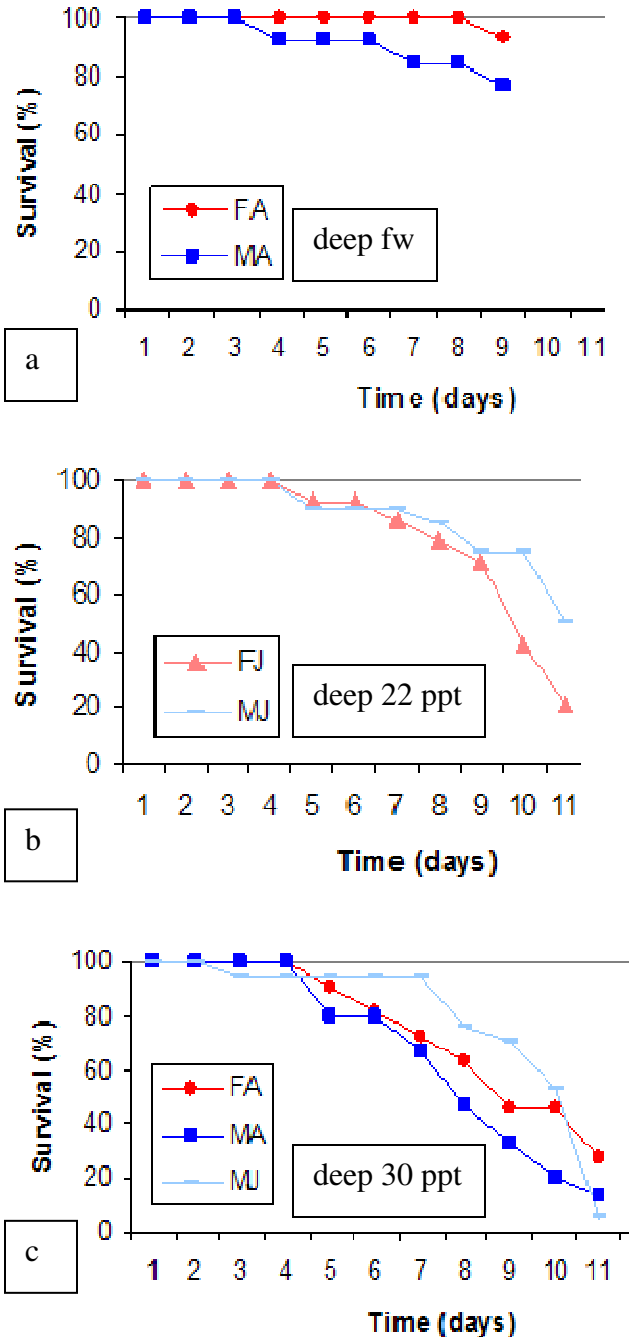


Fig. 3.7 Percent survival of *E. dugasti* over 11 days in deep water at 3 different salinities (a, FW; b, 22 ppt and c - 30 ppt).

3.2 *Sayamia germaini*

3.2.1 *Hemolymph osmolality in deep and shallow water at different salinities*

The hemolymph osmolality of adult (male and female) control groups (384 to 444 mOsm/kg) was hyperosmotic to shallow fresh water (26 to 50 mOsm/kg, Fig. 3.8a). Male adults held in shallow sea water (15 ppt, 374 to 400 mOsm/kg) for up to 9 days had hyperosmotic hemolymph (473 to 575 mOsm/kg). Male adults held in deep sea water (22 ppt, 543 to 610 mOsm/kg) were either hyperosmotic or isosmotic to the medium, while the hemolymph of female adults in deep water of the same concentration were always hyposmotic to the medium, but exhibited a wide range of hemolymph osmolalities (Fig. 3.8b). The hemolymph of male and female adults in deep full strength sea water (30 ppt, 770 to 824 mOsm/kg) was generally hyposmotic, but exhibited a range close to being isosmotic with the sea water (Fig. 3.8c, males after 9 days).

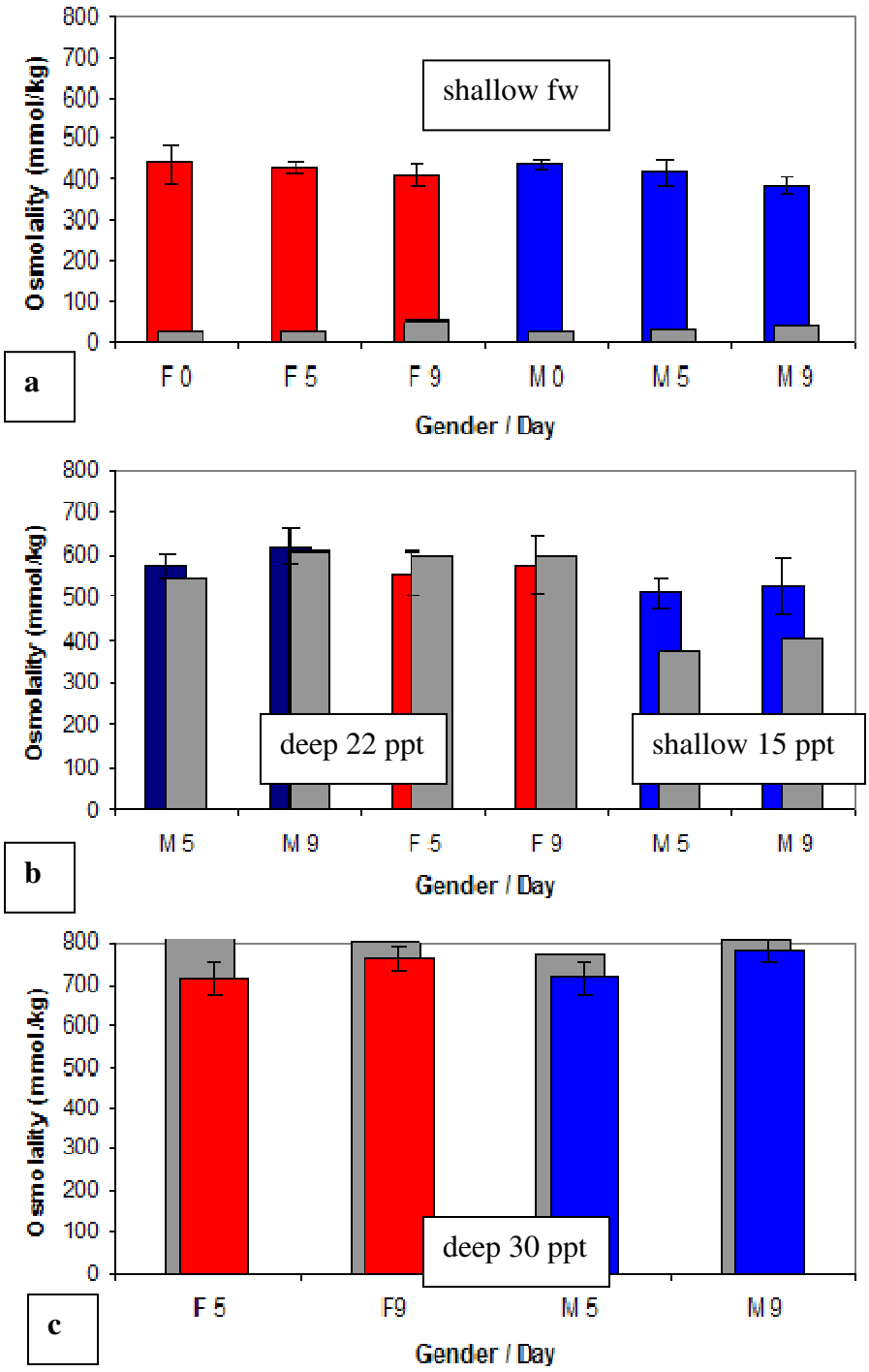


Fig. 3.8 Hemolymph osmolality of *S. germaini* held in shallow fresh water, 15 ppt, and in deep water at 22 and 30 ppt. Colored bars represent hemolymph osmolality, error bars = standard deviation, grey bars = water osmolality. The x-axis shows the number of days that crabs were subjected to a particular salinity. F = female, M = male, J = juvenile, number = # of days into experiment. a) Shallow fresh water. Red = ♀ adults n = 5, 4, 5, respectively, blue = ♂adults n = 5, 5, 5, respectively. b) Male adults held in shallow water at 15 ppt and female and male adults held in deep water at 22 ppt. Dark blue = ♂ adults n = 4, 4, respectively, held in 22 ppt, red = ♀ adult n = 4, 3, respectively, blue = ♂ adult n = 8, 8, respectively in 15 ppt sea water. c) Female and male adults in deep water at 30 ppt. Red = ♀ adult n = 10, 8, respectively, blue = ♂ adult n = 10, 6, respectively.

3.2.2 *Factors affecting hemolymph osmolality in shallow and deep water at different salinities*

The hemolymph osmolality of male and female adult *S. germaini* subjected to different salinities was significantly influenced by the length of exposure (in days) in combination with a particular salinity and increased (GLM - repeated measures ANOVA, p = 0.005, Tables C2, C3). The hemolymph osmolality between male and female groups (p = 0.00) was found to be significantly different. The hemolymph osmolality of those groups maintained in water at 0, 15, 22, and 30 ppt were all significantly different from each other (p = 0.00, Table 3.9).

Table 3.9 Homogenous subset after the post-hoc Tukey test of the repeated measures ANOVA using hemolymph osmolality data after 5 and 9 days of both male and female adult *S. germaini* subjected to different salinities. Means mOsm/kg for homogeneous groups are displayed. Based on Type I Sum of Squares. The error term is mean square (Error) = 984.397. The harmonic mean sample size 8.881 was used.

	salinity	***N***	***Subset***			
	ppt		1	2	3	4
Tukey HSD	0	9	407.667			
	15	8		518.688		
	22	7			582.857	
	30	14				742.071
	Sig.		1	1	1	1

3.2.3. *Survival in different salinities*

Water depth

Only water depth had a significant effect (change in -2LL if term removed from model: -2LL of water depth = 9.249, df=1, p= 0.002) on the survival after 5 days. The standard error was inflated (S.E. = 7338.199), so the Wald Statistic was not computed. Variables such as salinity, gender, the interaction of gender or water depth with salinity and the interaction of water depth with gender were not significant in the full model. The overall correct classification of the reduced model was 94.1%. However, only a little of

the variation in the odds of being alive after 5 days could be explained by the reduced model (Nagelkerke $R^2 = 0.288$, -2LL = 17.225* - final -2LL solution could not be found, as estimation terminated after maximum iterations had been reached (Table 3.10). After nine days into the experiment, no single factor (e.g., salinity, gender, water depth, or interaction of gender with salinity, water depth with salinity or water depth with gender) was found to be significant at $p = 0.05$.

Table 3.10 Variables in the reduced logistic regression equation of survival data of *S. germaini* after 5 days

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1	waterdepth1 shallow	-19.411	7338.199	0	1	0.998	0
	Constant	21.203	7338.199	0	1	0.998	1.62E+09

3.2.4 *Percent survival*

The percent survival of adult *S. germaini* over a period of 12 days is shown in Figure 3.9. The control animals kept in shallow fresh water (0.8 to 1.2 ppt) had the highest survival rate after 12 days. The survival of male adults in deep sea water at 22 ppt decreased after 5 days, while the survival of the males in deep water at 30 ppt decreased after 6 days and the survival of females subjected to deep water at 30 ppt sea water decreased after 7 days, while females subjected to deep sea water at 22 ppt decreased most rapidly after 10 days compared to the other groups. An increased relative risk (RR) of dying compared to the control (fresh water) was found for all groups after 9 days (MA 15 ppt, RR = 30; MA 22 ppt, RR = 20; FA 30 ppt, RR = 20, MA 30 ppt, RR = 30) except for female adults in deep water at 22 ppt (Table C8).

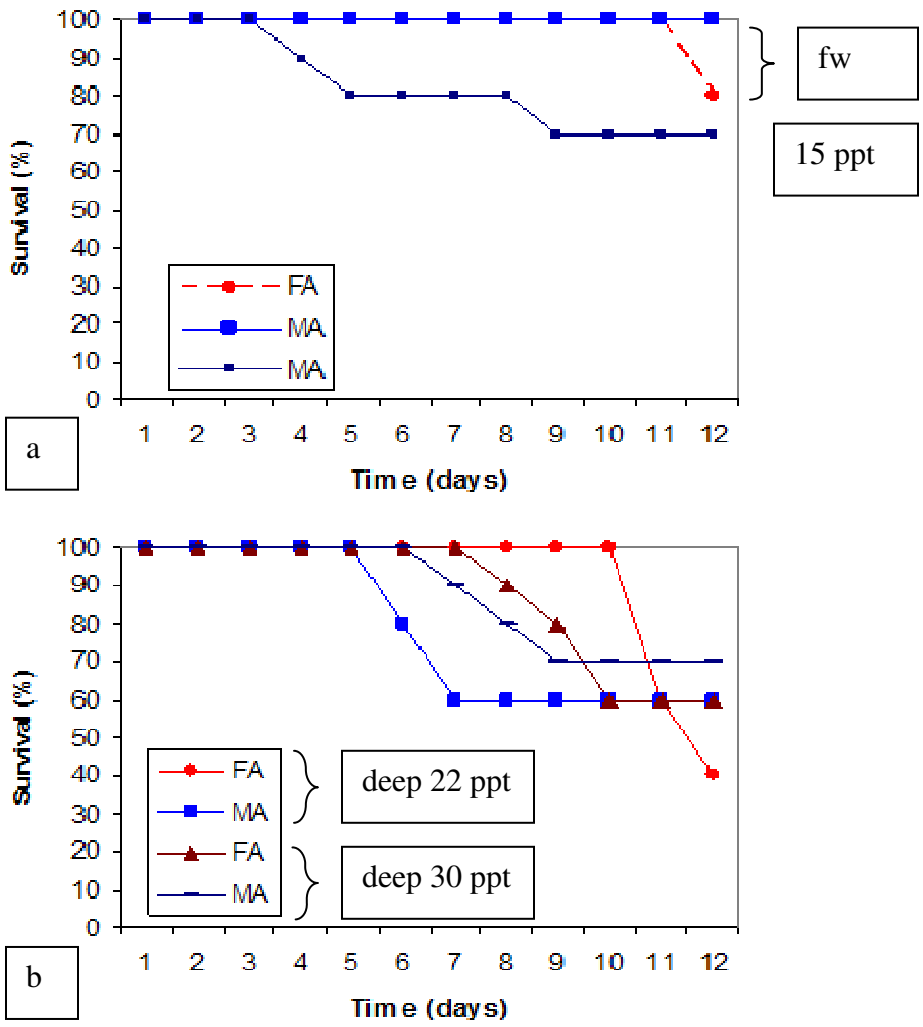


Fig. 3.9 Survival of adult *S. germaini* in shallow water of two salinities (fresh water and 15 ppt) and in deep water of two salinities (22 and 30 ppt). a) crabs held in shallow fresh water (n = 5 for 100%) and water at 15 ppt (n = 10 for 100%). b) crabs held in deep water at 22 ppt (females n = 4 for 100%, males n = 5 for 100%) and at 30 ppt (both genders n = 10 for 100%).

3.3 *Potamon smithianum*

3.3.1 *Hemolymph osmolality in different salinities*

The hemolymph osmolality of adult and juvenile control groups held in shallow fresh water (314 to 541 mOsm/kg) was found to be hyperosmotic to freshwater (18 to 53 mOsm/kg; Figure 3.10). There was a wide range of hemolymph osmolalities measured, especially among adult males after 1 and 13 days, juvenile males after 1 day, and female juveniles after 5 days. Female adult and male and female juveniles held in shallow sea water at 22 ppt (550 to 677 mOsm/kg) for up to 13 days had a mean hemolymph osmolality that was hyposmotic to the water but had some variation, while the mean hemolymph osmolality of male adults (664.5 mOsm/kg) after 9 days in shallow water at 22 ppt was higher than the osmolality of the water (592.3 mOsm/kg; Fig. 3.11). 'Mini' crabs in shallow sea water at 22 ppt survived for 27 days and their hemolymph was hyperosmotic to the water, but there was a great deal of variation (606 ± 120 mOsm/kg, Fig. 3.12). The mean hemolymph osmolality of female and male adults was hyposmotic to the shallow sea water (28 ppt, 725 to 916 mOsm/kg) after 5 and 9 days, but hyperosmotic after 13 days, when the sea water ranged from 697 to 707 mOsm/kg (Fig. 3.12).

When juvenile crabs were in deep water at 22 ppt their hemolymph osmolality was hyposmotic after 5 days, but hyperosmotic after 9 days, while the water osmolality remained stable throughout (556 and 573 mOsm/kg, respectively). Male adult crabs held in deep water at 22 ppt (560 mOsm/kg) maintained a hemolymph osmolality that was slightly hyper-osmotic over 9 days. A baseline hemolymph osmolality value measured

before the start of the experiment showed that crabs started out hyperosmotic to fresh water and already had a variable hemolymph osmolality (Fig. 3.13).

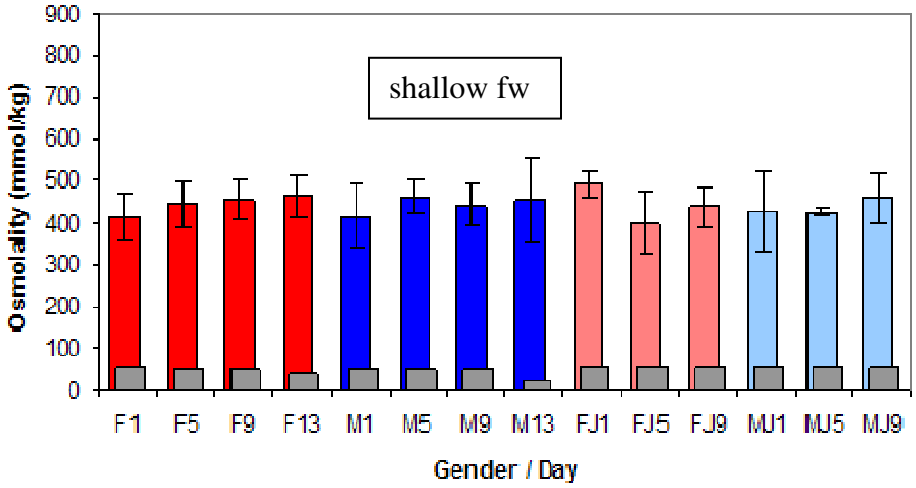


Fig. 3.10 Hemolymph osmolality of *P. smithianum* held in shallow fresh water. Colored bars represent hemolymph osmolality, error bars = standard deviation, grey bars represent water osmolality. The x-axis shows the number of days that crabs were subjected to a particular salinity. F = female, M = male, J = juvenile, number = # of days into experiment. Red = ♀ adult n = 10, 8, 8, 6 respectively, blue = ♂ adult n = 10, 7, 7, 6, respectively, pink = ♀ juvenile n = 5, 3, 2, respectively, and light blue = ♂ juvenile n = 5, 2, 1, respectively.

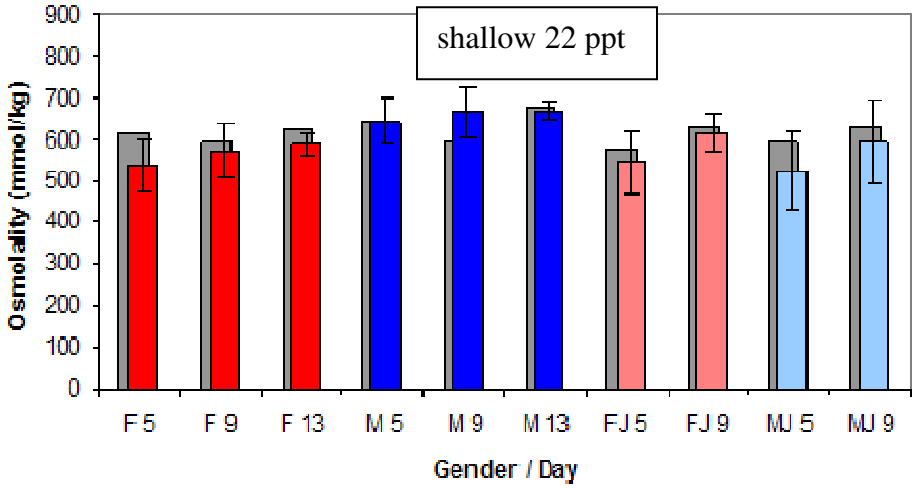


Fig. 3.11 Hemolymph osmolality of *P. smithianum* held in shallow water at 22 ppt. Colored bars represent hemolymph osmolality, error bars = standard deviation, grey bars represent water osmolality. The x-axis shows gender and day the hemolymph was sampled. F = female, M = male, J = juvenile, number = # of days into experiment. Red = ♀ adult n = 12, 8, 7, respectively, blue = ♂ adult n = 4,4, 3, respectively, pink = ♀ juvenile n = 6, 2, respectively; and light blue = ♂ juvenile n = 6, 4, respectively.

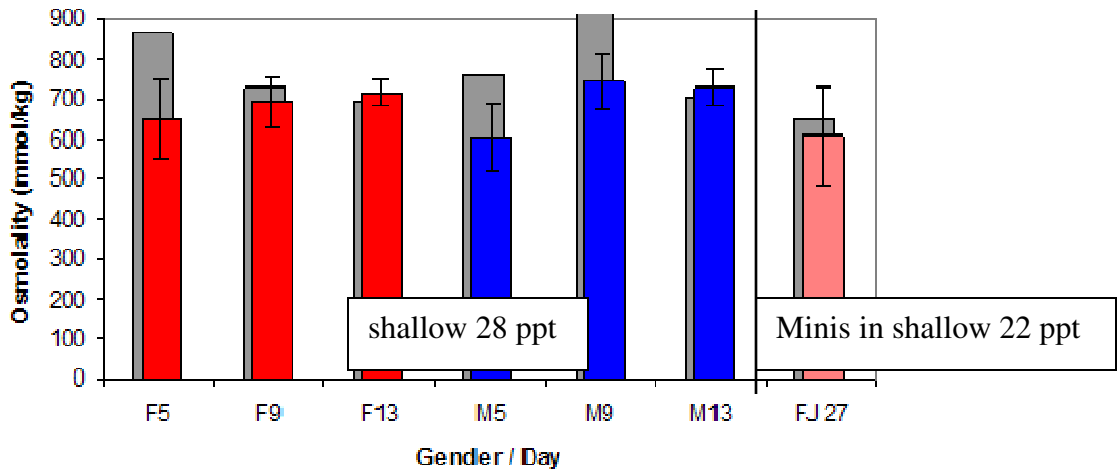


Fig. 3.12 Hemolymph osmolality of *P. smithianum* held in shallow water at 28 and 22 ppt. Colored columns represent hemolymph osmolality, error bars = standard deviation, grey columns represent water osmolality. The x-axis shows the number of days that crabs were subjected to a particular salinity. F = female, M = male, J = juvenile, number = # of days into experiment. Red = ♀ adults n = 13, 12, 11, respectively, blue = ♂ adults n = 9, 8, 7, respectively, in 28 ppt and pink = ♀ juvenile n = 7 after 27 days in 22 ppt .

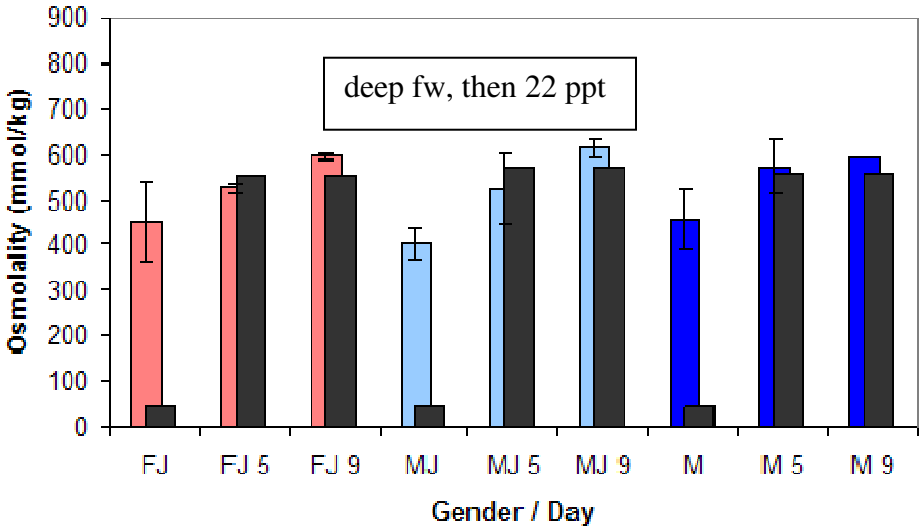


Fig. 3.13 Hemolymph osmolality of *P. smithianum* held in deep fresh water and then in deep water at 22 ppt. Colored columns represent hemolymph osmolality, error bars = standard deviation, grey columns = water osmolality. The x-axis shows the on which days the hemolymph was sampled. F = female, M = male, J = juvenile, number = # of days into experiment, if no number, then it was sampled before the start of the experiment as a baseline. Pink = ♀ juveniles n = 5, 2, 2, respectively, light blue = ♂ juveniles n = 5, 5, 3, respectively; and blue = ♂ adults n = 5, 5, 1, respectively.

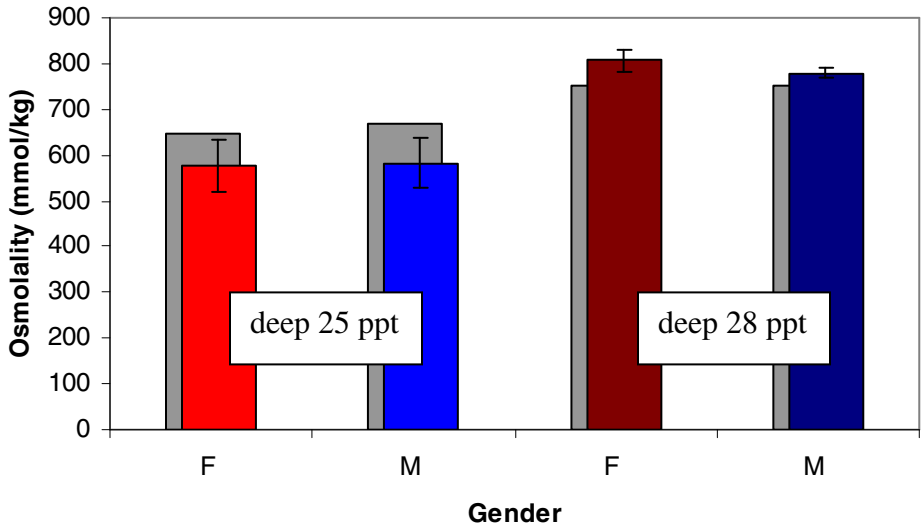


Fig. 3.14 Hemolymph osmolality of *P. smithianum* held in deep water at 25 and 28 ppt after 5 days. Colored columns represent hemolymph osmolality, error bars = standard deviation, grey columns represent water osmolality. The x-axis shows the gender of the crabs subjected to a particular salinity. F = female, M = male, J = juvenile. Red = ♀adult n = 9, blue = ♂ adult n = 5 in sea water at 25 ppt, brown = ♀ adults n = 3, and dark blue = ♂ adults n = 3 in deep sea water at 28 ppt.

Male and female adult crabs held in deep water at 25 ppt (647 to 669 mOsm/kg), had a hemolymph osmolality that was hyposmotic to the water, while crabs in deep water at 28 ppt (751 mOsm/kg) had a higher hemolymph osmolality than that of the sea water (hyperosmotic; Fig. 3.14).

3.3.2 *Factors affecting hemolymph osmolality in shallow and deep water at different salinities*

The duration (in days) (GLM-repeated measures ANOVA, p = 0.00) of the experiment significantly increased hemolymph osmolality of juvenile and adult *P. smithianum* held in shallow water, and the number of days of exposure to a particular salinity (p = 0.04) also significantly increased hemolymph osmolality (Tables D2, D3). Hemolymph osmolality between groups held in different salinities (fresh water, 22 and 28 ppt) was significantly different (p = 0.00, Tables 3.11, D4, D5).

Table 3.11 Homogenous subsets of post-hoc Tukey test of repeated measures ANOVA of hemolymph osmolality data after 5, 9 and 13 days for juvenile and adult *P. smithianum* in shallow water at three different salinities. Means mOsm/kg for homogeneous groups are displayed. Based on Type I sum of squares. The error term is mean square (Error) = 1821.370. Harmonic mean sample size of 12.203 was used.

	salinity	**N**	**subset**		
	ppt		1	2	3
Tukey HSD(a,b)	0	12	447.77		
	22	10		587.03	
	28	16			683.5
	Sig.		1	1	1

Effect of salinity and water depth

Salinity (univariate ANOVA, p = 0.00) and the interaction of water depth with salinity (p = 0.02) had significant effects on the hemolymph osmolality of juvenile and adult *P. smithianum* after 5 days (Table D7), lower hemolymph osmolality was found animals held in deep rather than shallow water. Experimental groups held in 22 and 25 ppt, and in 25 and 28 ppt were in the same homogenous subset of the post-hoc Tukey test and therefore did not have a significantly different hemolymph osmolality after 5 days (Table 3.12).

Table 3.12 Homogenous subsets of the Tukey test of the univariate ANOVA of hemolymph osmolality of juvenile and adult *P. smithianum* in shallow and deep water of different salinities after 5 days. Means mOsm/kg for homogeneous groups are displayed. Based on Type I sum of squares. The error term is mean square (Error) = 5050.215. The group sizes are unequal. The harmonic mean of the group sizes, 9.895, is used. Type I error levels are not guaranteed.

	salinity	**N**	**subset**			
	ppt		1	2	3	4
Tukey HSD	0	35	439.66			
	22	33		555.30		
	25	14		578.21	578.21	
	28	24			645.46	
	30	3				779.33
	Sig.		1	0.952	0.227	1

Effect of water depth after 5 days

After 5 days, hemolymph osmolality was significantly lower in male and female adults in shallow 28 ppt water compared to deep water (Table 3.13).

Table 3.13 Summary of t-tests that showed significant differences in hemolymph osmolality between *P. smithianum* subjected to shallow or deep water after 5 days. Equal variances assumed.

			t- test for Equality of Means						
			t	**df**	**Sig. (2-tailed)**	**Mean difference**	**Std. Error Difference**	**95% CI of the difference**	
Water depth	Gender/ age	salinity						lower	upper
deep vs shallow	Female adults	28 ppt	- 2.659	14	0.019*	- 156.41	58.82	- 282.57	- 30.24
deep vs shallow	Male adults	28 ppt	- 3.46	10	0.006*	- 176.11	50.89	- 289.51	- 62.70

3.3.3 *Survival in different salinities*

Effect of salinity on survival after 5 days

Binary forward stepwise logistic regression found that salinity regime had a significant effect on survival after 5 days (if salinity was removed from the model, change in -2LL of salinity = 4.479, df = 2, p = 0.034). Variables such as water depth and age were not significant in the full model. The correct overall classification of the reduced model was 87.1%. However, only a small amount of the variation associated with the log odds of being alive could be explained by this reduced model (Nagelkerke $R^2 = 0.054$, -2LL = 108.854, Table 3.14).

Table 3.14 Variables in the reduced logistic regression equation of survival data of *P. smithianum* after 5 days

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1	salinity	.047	.023	4.263	1	.039 *	1.048
	Constant	1.340	.339	15.647	1	.000 *	3.818

Effect of age on survival after 9 and 13 days

Binary stepwise forward logistic regression found that age had a significant effect on survival after 9 days (if age was removed from the model, change in -2LL of age = 12.337, df = 2, p = 0.002). Variables such as salinity, water depth and the interaction of water depth with salinity were not significant in the full model. The overall correct percentage of classification was 67.7% for the reduced model, however, only a small amount of the variation associated with the log odds of being alive could be explained by this reduced model (Nagelkerke R^2 = 0.128, -2LL = 153.210, Table 3.15).

Table 3.15 Variables in the reduced logistic regression equation of survival data of *P. smithianum* after 9 days

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1	age			11.665	2	.003 **	
	age1 juvenile	-.833	.564	2.178	1	.140	.435
	age2 adult	.653	.536	1.483	1	.223	1.922
	constant	.405	.456	.789	1	.374	1.500

Binary logistic regression also found that age had a significant effect on survival after 13 days (if age was removed from the model, change in -2LL of age = 26.644, df = 2, p = 0.00). The variable salinity and the interaction of water depth with salinity were not significant in the full model. The correct overall classification of the reduced model was 69.9%, however, only a small amount of the variation associated with the log odds of being alive could be explained by this reduced model (Nagelkerke R^2 = 0.289, -2LL = 117.497, Table 3. 16). After 5 days, salinity regime had a significant effect on survival, but after 9 and 13 days only age had a significant effect.

Table 3.16 Variables in the reduced logistic regression equation of survival data of *P. smithianum* after 13 days

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1	age			14.946	2	.001	
	age1 juvenile	-2.552	.866	8.688	1	*.003	.078
	age2 adult	.492	.526	.876	1	.349	1.636
	constant	.201	.449	.199	1	.655	1.222

3.3.4 *Percent survival*

'Mini' crabs held in shallow water at 22 ppt had a 60% survival rate after 20 days (Fig. 3.15). Adult female and males in fresh water (0.22 to 1.2 ppt), had a 60% survival rate after 14 days, and all the juveniles in fresh water had died after 11 days (Fig. 3.16a). The relative risk (RR) of dying by being in an increased salinity compared to the control (fresh water) was found to be similar for most groups, except for crabs held in 28 ppt shallow sea water (FA, RR = 0.39 and MA, RR = 0.37) and for male adult crabs held in 25 ppt (RR = 0.03), where the relative risk of dying was lower than the control (Table D12).

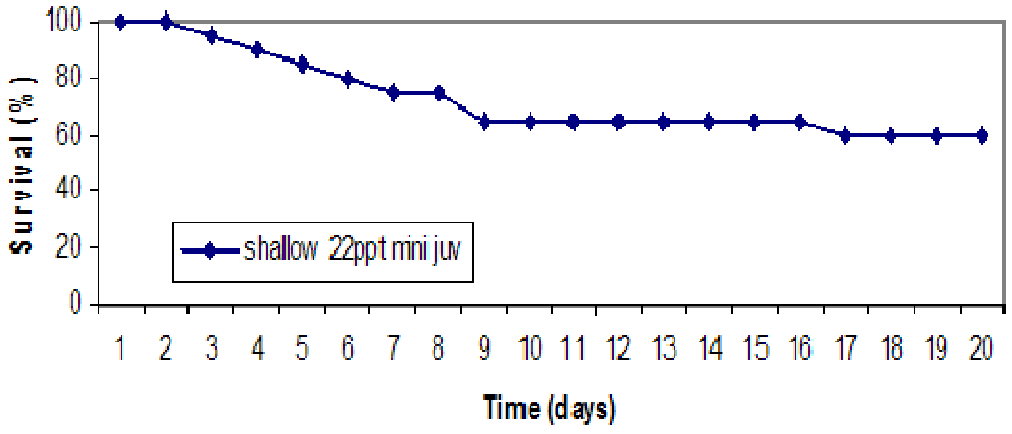


Fig. 3.15 Survival of mini juvenile *P. smithianum* in shallow water at 22 ppt over 20 days. n = 20 for 100 %.

Survival of *P. smithianum* in shallow water at 22 ppt was lowest in female juveniles (only 14.5% survived for 13 days), higher in male juveniles (50%) and highest in adults (60%). Eighty-four point five percent of female and 77.7% of male adults survived for 14 days in shallow water at 28 ppt (Fig. 3.16b). All adult males in deep water at 25 ppt survived for 9 days, as did 88.8 % of female adults. Only 54.5% of male and female adults survived in deep water at 30 ppt for 9 days.

Survival of female juvenile *P. smithianum* in deep fresh water (1.2 ppt) for 2 weeks was 28.5%, 75% in male juveniles and 71.4% in male and female adults (Fig. 3.17). When this group was transferred to deep water of 22 ppt for 10 days, all male juveniles died, 14% of female juveniles survived, as did 35% of adults.

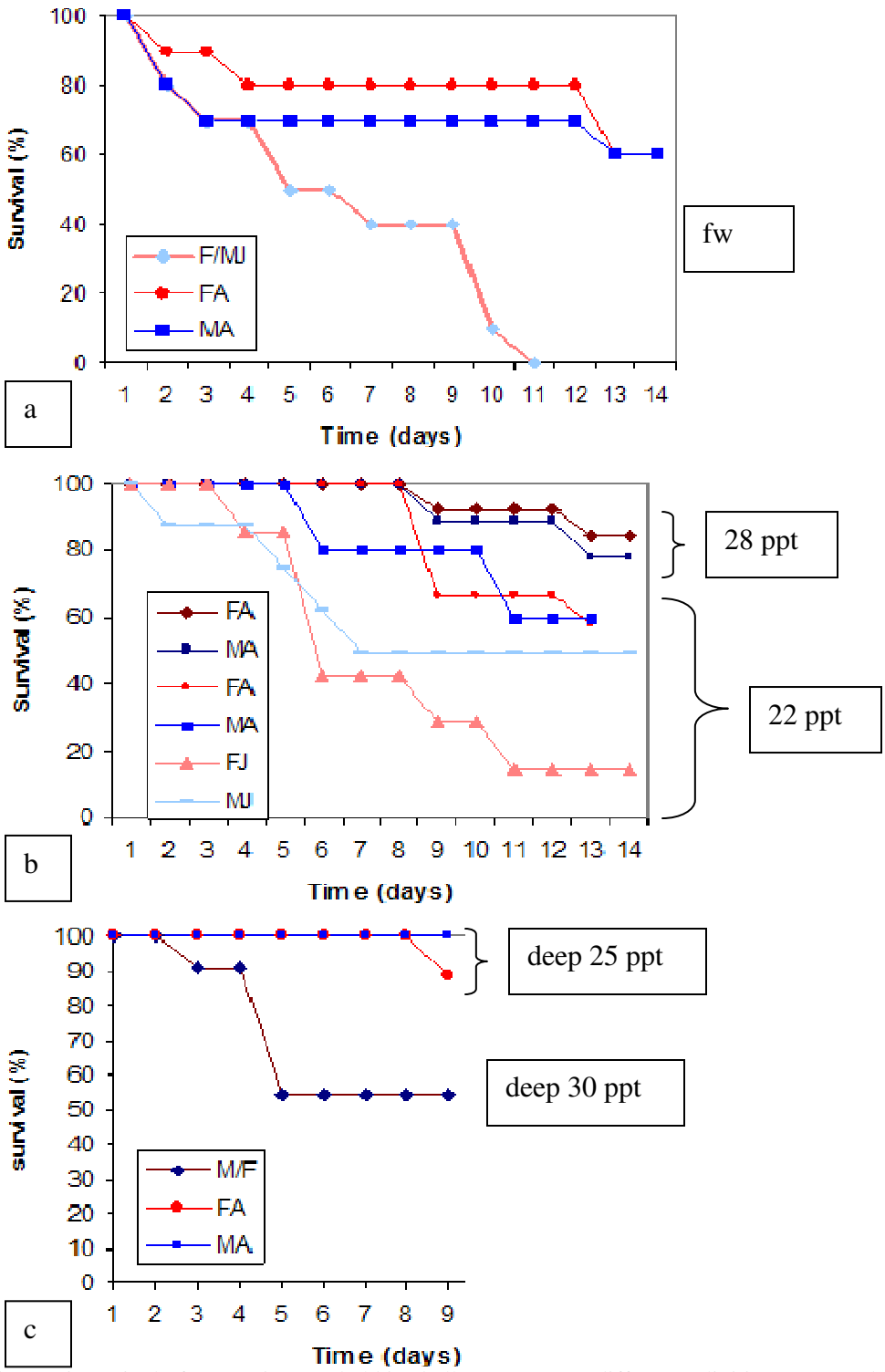


Fig. 3.16 Survival of *P. smithianum* held in shallow water at 3 different salinities (0, 22 and 28 ppt) over a 14 day period and in deep water at 25 and 30ppt over 9 days. a) Survival of adults and juveniles shallow fresh water, n=10 for each group. b) Survival of adults (n = 7, 8, respectively) in shallow water at 28 ppt, and adults (n = 12, 5, respectively) and juveniles (n = 13, 9, respectively) in water at 22 ppt. c) Survival of adults in deep water at 25 and 30 ppt over 9 days. Starting numbers were n = 11 for the 30 ppt group, n = 9 for the female 25 ppt group and n = 4 for the male 25 ppt group.

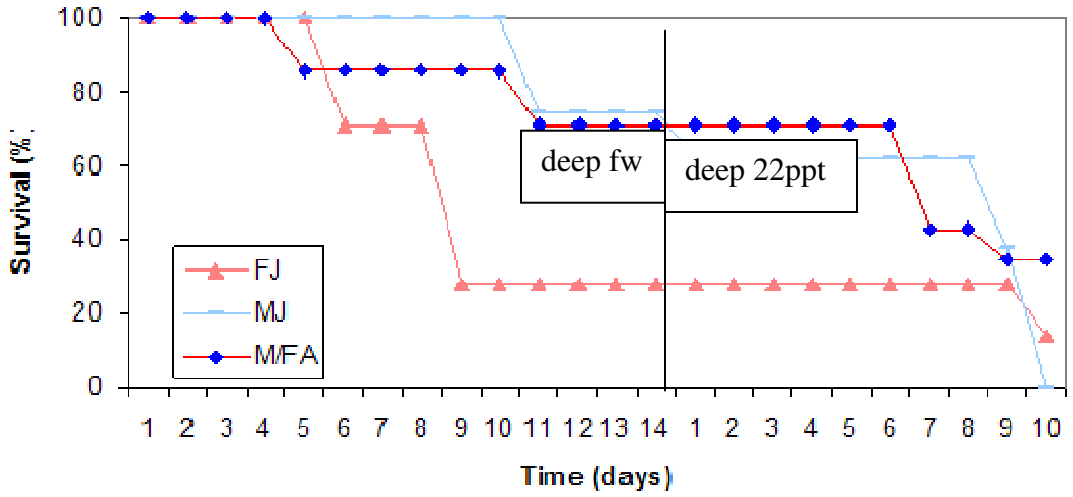


Fig. 3.17 Survival of *P. smithianum* in deep fresh water (0 ppt) for 14 days and then in deep water at 22 ppt for 10 days. Female juvenile and combined adult groups had starting numbers of n = 7, while the male juvenile group had starting numbers of n = 8.

3.4 *Larnaudia chaiyaphumi*

3.4.1 *Hemolymph osmolality in shallow water of different salinities*

The mean hemolymph osmolality of 'mini' juveniles after 12 days in fresh water was hyperosmotic (328 to 462 mOsm/kg), hyper-/iso-/hyposmotic (416 to 652 mOsm/kg) in water at 22 ppt, and hyposmotic (590 tp 884 mOsm/kg) in full strength sea water (30 ppt). The hemolymph osmolality variation increased with increasing water salinity (Fig. 3.18).

The hemolymph osmolality of juveniles and adults (335 to 488 mOsm/kg, mean 436 mOsm/kg) was hyperosmotic in shallow fresh water (Fig. 3.19). The hemolymph osmolality of adults and juveniles held in water at 22 ppt showed a wide range of values and was both hyper- and hyposmotic (Fig. 3.20).

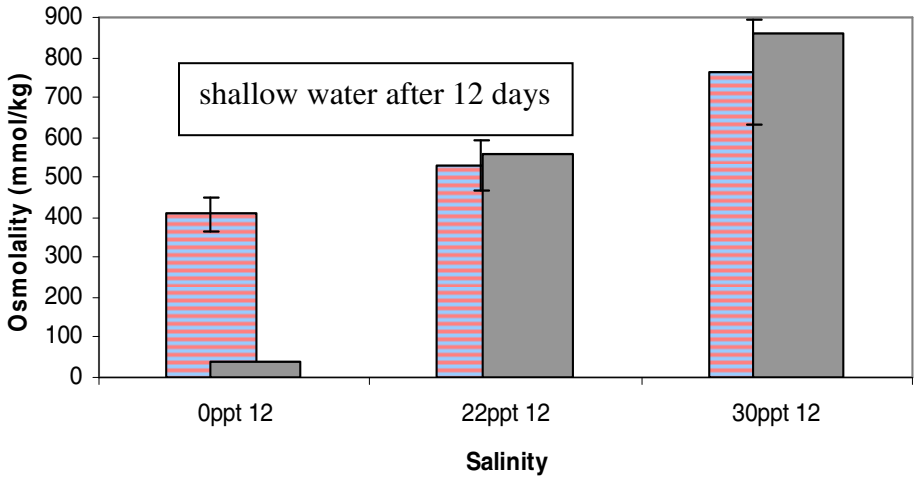


Fig. 3.18 Hemolymph osmolality of mini *L. chaiyaphumi* juveniles in shallow water at 0, 22 and 30 ppt was measured after 12 days. The striped columns represent hemolymph osmolality, error bars = standard deviation, the number = # of days into the experiment. Fresh water (0 ppt, n = 8), 22 ppt (n=10) and 30 ppt (n=6). The grey column shows measured water osmolality.

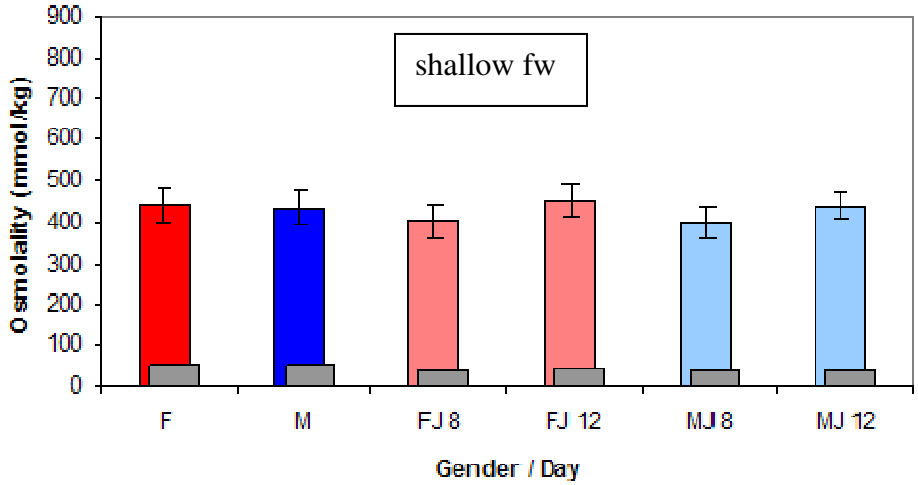


Fig. 3.19 Hemolymph osmolality of *L. chaiyaphumi* held in shallow fresh water. Red, blue, pink and light blue colored columns represent hemolymph osmolality, error bars = standard deviation. F = female, M = male, J = juvenile, number = # of days into experiment. Grey columns show measured water osmolality. Red = ♀ adults baseline n = 7, blue = ♂ adults baseline n = 7, pink = ♀ juveniles n = 5, 4, respectively; light blue = ♂ juveniles n = 6, 5, respectively.

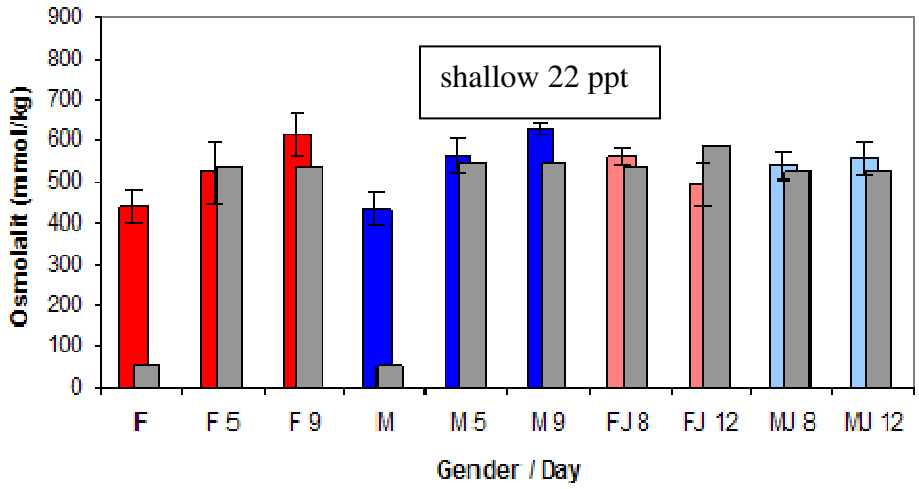


Fig. 3.20 Hemolymph osmolality of *L. chaiyaphumi* held in shallow water at 22 ppt. Red, blue, pink and light blue columns represent hemolymph osmolality, error bars = standard deviation. F = female, M = male, J = juvenile, number = # of days into experiment. Grey columns show measured water osmolality. Red = ♀ adult n = 7, 6, 3, respectively, and blue = ♂ adult n = 7, 6, 4, respectively, in shallow fresh water and then in 22 ppt. Pink = ♀ juvenile n = 10, 5, respectively, and light blue = ♂ juvenile n = 11, 3, respectively, in water at 22 ppt.

3.4.2 *Factors affecting hemolymph osmolality in shallow and deep water at different salinities*

The hemolymph osmolality of *L. chaiyaphumi* held in shallow water at 22 ppt for 5 and 9 days significantly increased with the length of exposure (in days) (GLM–repeated measures ANOVA, p = 0.01) (Tables E1, E2) and multiple comparisons showed that each day had significantly different hemolymph osmolality averages (Tables 3.17, E5).

Table 3.17 Homogenous subset of the post-hoc Tukey Tests after the repeated measures ANOVA of hemolymph osmolality data of *L. chaiyaphumi* in water at 22 ppt after 5 and 9 days and a covariate in fresh water. Means mOsm/kg for homogeneous groups are displayed. The group sizes are unequal. The harmonic mean of the group sizes, 10.080, is used. Type I error levels are not guaranteed.

	group	***N***	Subset for alpha = .05		
			1	2	3
Tukey HSD	covariate	14	436.2857		
	day 5	12		542.4166	
	day 9	7			624.4285
	Sig.		1	1	1

Hemolymph osmolality data of male and female juvenile *L. chaiyaphumi* in shallow water of different salinities (0 and 22 ppt) after 8 and 12 days was significantly affected by various interactions: length of exposure (in days) combined with salinity (p = 0.01), and length of exposure (in days) combined with gender and salinity (p = 0.01, Tables E6, E7). Hemolymph osmolality was found to be significantly higher in high salinity compared to low salinity (p = 0.00; Table E8).

Effect of salinity after 13 days

There was no significant difference in hemolymph osmolality between males and females within the same salinity. Hemolymph osmolality of pooled male and female juvenile and mini *L. chaiyaphumi* in different salinities (0, 22 and 30 ppt) was significantly different in 0, 22 and 30 ppt water, with hemolymph osmolality significantly increasing with increasing salinity (Univariate ANOVA, p = 0.00) after 13 days (Tables 3.18, E12).

Table 3.18 Homogenous subsets of post-hoc Tukey test after the Univariate ANOVA of hemolymph osmolality data after 13 days of juvenile and mini juvenile *L. chaiyaphumi* in different salinities (fresh water, 22 and 30 ppt), with pooled gender. Means mOsm/kg for homogeneous groups are displayed. Based on Type I sum of squares. The error term is mean square (Error) = 4578.804. The group sizes are unequal. The harmonic mean of the group sizes, 10.674, is used. Type I error levels are not guaranteed.

	salinity	***N***	***Subset***		
Tukey HSD	ppt		1	2	3
	0	17	426.35		
	22	18		525.22	
	30	6			764.5
	Sig.		1	1	1

3.4.3 *Survival in different salinities*

The logistic regression procedure was not able to produce a model to explain the survival of the crabs in different levels of salinity after 5 or 9 days (Table E14).

Binary stepwise forward logistic regression found that age had a significant effect (change in -2LL if age removed from model: -2LL of age = 11.054, df2, p = 0.004) on survival after 13 days (Tables 3.36, 3.37). The factor salinity was not significant for the full model. The overall correct classification of the reduced model was 65.8%, and only a small amount of the variation associated with the log odds of being alive after 13 days could be explained by the reduced model (Nagelkerke R^2 = 0.181, -2LL = 86.995, Table 3.19).

Table 3.19 Variables in the reduced logistic regression equation of survival data of *L. chaiyaphumi* on day 13

		B	S.E.	Wald	df	Sig.	Exp(B)
Step 1	age			8.73	2	0.01*	
	age1 juvenile	-1.87	0.64	8.63	1	0.00*	0.15
	age2 adult	-1.54	0.79	3.73	1	0.05*	0.22
	Constant	1.87	0.54	12.15	1	0.00*	6.50

3.4.4 *Percent survival*

Female and male juvenile crabs held in shallow fresh water (the control groups) survived equally well after 13 days (66%, Fig. 3.21a). Thirty-three percent of male juveniles compared to 50% female juveniles survived for 13 days in shallow water at 22 ppt (Fig. 3.21b). All 'mini' juveniles held in shallow water at 22 ppt survived over a 13 day period, 90% of those held in fresh water survived, as did 70% of those held in 30 ppt (Fig. 3.22). Male adults held in shallow water at 22 ppt had a higher survival rate (66%) than females (50%, Fig. 3.23), however the relative risk of dying was similar (Table E17).

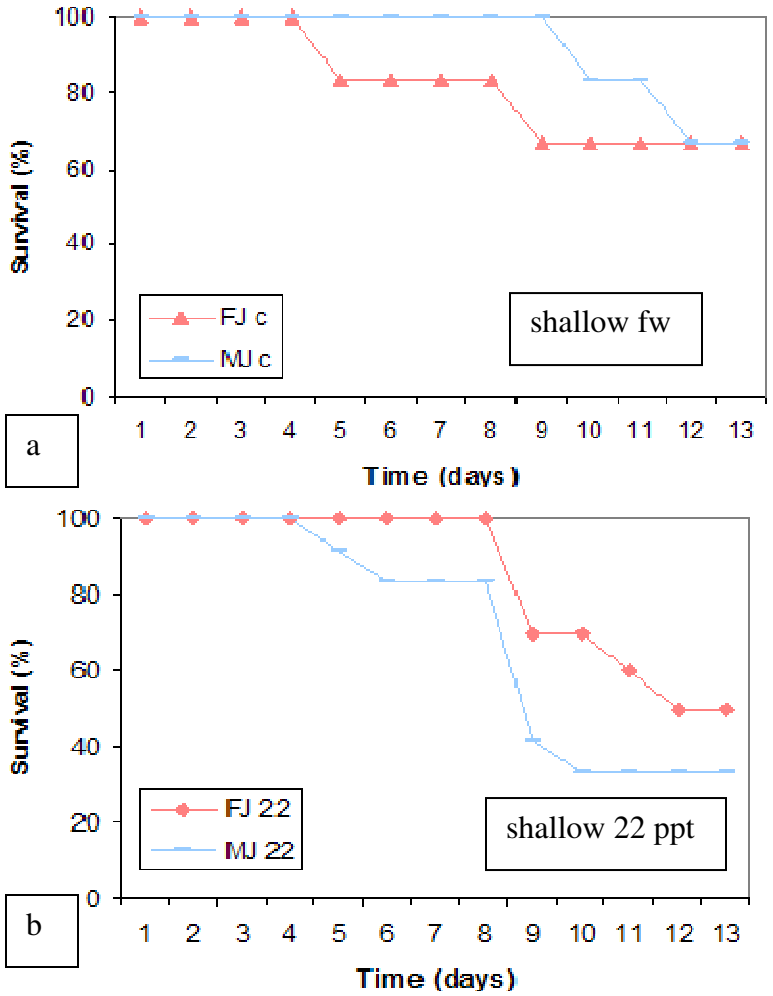


Fig. 3.21 Survival of juvenile *L. chaiyaphumi* held in shallow water of two different salinities (fresh water and 22 ppt). a) Juvenile crabs held in fresh water (n = 6). b) Juvenile crabs held in 22 ppt. Females (n = 10), males (n = 12).

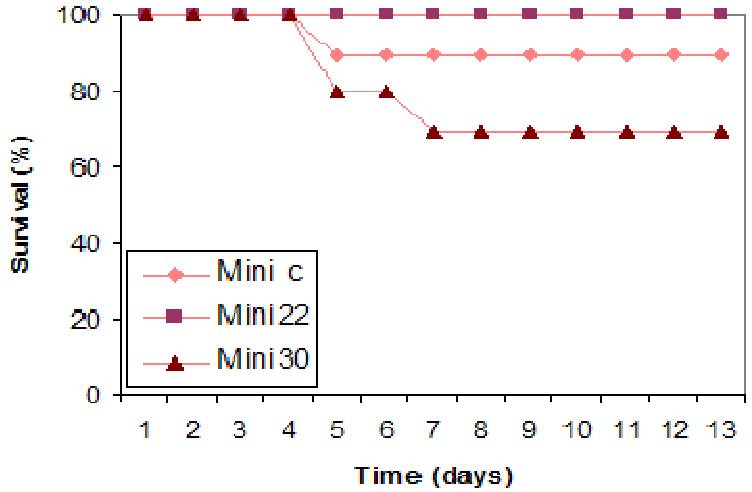


Fig. 3.22 Survival of 'mini' juvenile *L. chaiyaphumi* held in shallow water at 3 different salinities (fresh water, 22 and 30 ppt). n = 10 for each group.

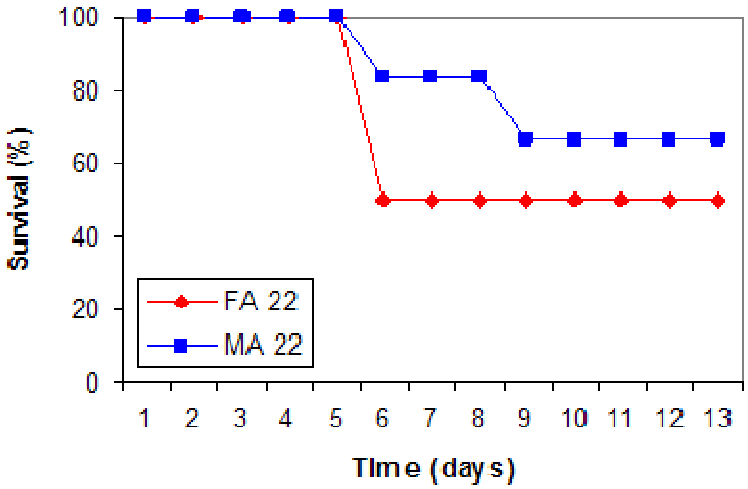


Fig. 3.23 Survival of adult *L. chaiyaphumi* held in shallow water at 22 ppt. n = 6 for each group.

3.5 Comparison of species and families

3.5.1 *Comparison of osmolality data*

Hemolymph osmolality for crabs of different age groups, water depths, and gender were pooled. One-way ANOVAs of the control groups in fresh water (days 5, 9 and 13), of the groups in 22 ppt (days 9 and 13), and of the groups in 30 ppt (days 5, 9 and 13) revealed that in most cases at least one species within each group was significantly different from the other species (Table 3.20).

Table 3.20 One-way ANOVA results of comparing species hemolymph osmolalities at three salinities over time (* = significantly different).

	Oneway ANOVA	**F**	**Sig**	**Species compared**
Control 0 ppt	day 1	0.08	0.92	*S. germaini, P. smithianum, L. chaiyaphumi*
	day 5	16.78	0.00 *	*E. dugasti, S. germaini, P. smithianum*
	day 9	8.45	0.00 *	*E. dugasti, S. germaini, P. smithianum, L. chaiyaphumi*
	day 13	27.78	0.00 *	*E. dugasti, P. smithianum, L. chaiyaphumi*
22 ppt	day 5	0.36	0.78	*E. dugasti, S. germaini, P. smithianum, L. chaiyaphumi*
	day 9	13.19	0.00 *	*E. dugasti, S. germaini, P. smithianum, L. chaiyaphumi*
	day 13	17.00	0.00 *	*E. dugasti, P. smithianum, L. chaiyaphumi*
30 ppt	day 5	3.58	0.03 *	*E. dugasti, S. germaini, P. smithianum*
	day 9	3.54	0.04 *	*E. dugasti, S. germaini, P. smithianum*
	day 13	4.45	0.02 *	*E. dugasti, P. smithianum, L. chaiyaphumi*

No significant difference was found between the hemolymph osmolality of the parathelphusid (*S. germaini*) and of the potamids (*P. smithianum* and *L. chaiyaphumi*) bled on the first day of the experiment. After 5 days in fresh water the hemolymph osmolality of the parathelphusid *E. dugasti* was significantly lower than that of the other two species (one parathelphusid and one potamid) held in fresh water for 5 days. After 9 days in fresh water the hemolymph osmolality of *E. dugasti* stayed almost the same, but that of *S. germaini* and *L. chaiyaphumi* was lower than on day one but the difference was not found to be significant. After 13 days in fresh water *E. dugasti* had a significantly lower hemolymph osmolality than *L. chaiyaphumi* in fresh water (which had increased, Table 3.21). In the case of the potamid *P. smithianum* the hemolymph osmolality did not change significantly within the first 9 days in fresh water, but after this it was significantly higher than all the other three species in fresh water.

There was no significant difference in the hemolymph osmolality between species or families after 5 days in sea water of 22 ppt. After 9 days in sea water of 22 ppt the hemolymph osmolality of the parathelphusid *E. dugasti* was significantly lower than that of the other species. After 13 days in sea water at 22 ppt *E. dugasti* had the highest hemolymph osmolality, similar to *P. smithianum.* These two species belong to different families and both had a significantly higher hemolymph osmolality than *L. chaiyaphumi* (Table 3.22).

Comparing the hemolymph osmolality data of different species subjected to water at 30 ppt revealed that the potamid *P. smithianum* always had the lowest hemolymph osmolality. After 5 and 9 days in 30 ppt sea water *P. smithianum* had a significantly lower hemolymph osmolality than that of the other potamid *L. chaiyaphumi*, while the

parathelphusid *E. dugasti* had an intermediate hemolymph osmolality, which was not significantly different from either potamid (Table 3.24). After 13 days in sea water at 30 ppt, no significant differences were found among the hemolymph osmolality of the three species (Table 3.23).

Table 3.21 Homogenous Subset after a Tukey test of pooled species hemolymph osmolalities when subjected to 0 ppt for 1, 5, 9 and 13 days. Means mOsm/kg for homogeneous groups are displayed. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

DAY 1			
species	**N**	**Subset for alpha = .05**	
		1	
P. smithianum	30	430.03	
L. chaiyaphumi	14	436.29	
S. germaini	10	437	
Sig.		0.94 NS	
Harmonic mean sample size	14.651		
Day 5			
species	**N**	**Subset for alpha = .05**	
		1	**2**
E. dugasti	51	385.27	
S. germaini	8		422.5
P. smithianum	20		440.95
Sig.		1	0.37 NS
Harmonic mean sample size	15.416		
Day 9			
species	**N**	**Subset for alpha = .05**	
		1	**2**
E. dugasti	42	394.14	
S. germaini	9	398.67	
L. chaiyaphumi	11	398.82	
P. smithianum	19		448.53
Sig.		0.99	1
Harmonic mean sample size	14.365		
Day 13			
species	**N**	**Subset for alpha = .05**	
		1	**2**
E. dugasti	34	358.62	
L. chaiyaphumi	17		426.35
P. smithianum	12		459.75
Sig.		1	0.08 NS
Harmonic mean sample size	17.486		

Table 3.22 Homogenous Subset after a Tukey test of pooled species hemolymph osmolalities when subjected to 22 ppt for 5, 9 and 13 days. Means mOsm/kg for homogeneous groups are displayed. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

DAY 5			
Species	**N**	**Subset for alpha = .05**	
		1	
L. chaiyaphumi	11	535.09	
E. dugasti	20	543.95	
P. smithianum	28	551.89	
S. germaini	8	564.63	
Sig.		0.66 NS	
Harmonic mean sample size	13.262		
DAY 9			
Species	**N**	**Subset for alpha = .05**	
		1	**2**
E. dugasti	34	506.68	
L. chaiyaphumi	28		570.21
S. germaini	7		601.43
P. smithianum	18		602.33
Sig.		1	0.45 NS
Harmonic mean sample size	15.178		
DAY 13			
Species	**N**	**Subset for alpha = .05**	
		1	**2**
L. chaiyaphumi	18	525.22	
P. smithianum	17		609.71
E. dugasti	27		627.59
Sig.		1	0.61 NS
Harmonic mean sample size	19.813		

No significant difference in hemolymph osmolality (after 5 days, $p = 0.29$; after 9 days, $p = 0.277$) was found between *E. dugasti* and *P. smithianum* (different families) subjected to sea water at 28 ppt.

Table 3.23 Homogenous Subset after a Tukey test of pooled species hemolymph osmolalities when subjected to sea water at 30 ppt for 5, 9 and 13 days. Means mOsm/kg for homogeneous groups are displayed. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

DAY 5			
species	**N**	**Subset for alpha = .05**	
		1	**2**
P. smithianum	28	665.54	
E. dugasti	48	705.83	705.83
L. chaiyaphumi	20		712.75
Sig.		0.09 NS	0.93 NS
Harmonic mean sample size	28.156		
DAY 9			
species	**N**	**Subset for alpha = .05**	
		1	**2**
P. smithianum	20	713.05	
E. dugasti	27	728.7037	728.7037
S. germaini	14		770
Sig.		0.722 NS	0.113 NS
Harmonic mean sample size	18.932		
DAY 13			
species	**N**	**Subset for alpha = .05**	
		1	
P. smithianum	18	721.8889	
L. chaiyaphumi	6	764.5	
E. dugasti	26	783.8846	
Sig.		0.084 NS	
Harmonic mean sample size	11.508		

3.5.2 *Comparison of survival within Parathelphusidae*

Survival was better in the control groups of adult *S. germaini* and *E. dugasti* compared to groups kept in water at 15 ppt (Fig. 3.24). *Sayamia germaini* held in deep sea water at 22 ppt showed a trend towards greater survival rate than *E. dugasti.* Males of both species survived longer than females after the initial 6 days (Fig. 3.25), and adult *S. germaini* had a higher percentage of survival in full strength sea water than sub-adult and juvenile *E. dugasti* (Fig. 3.26).

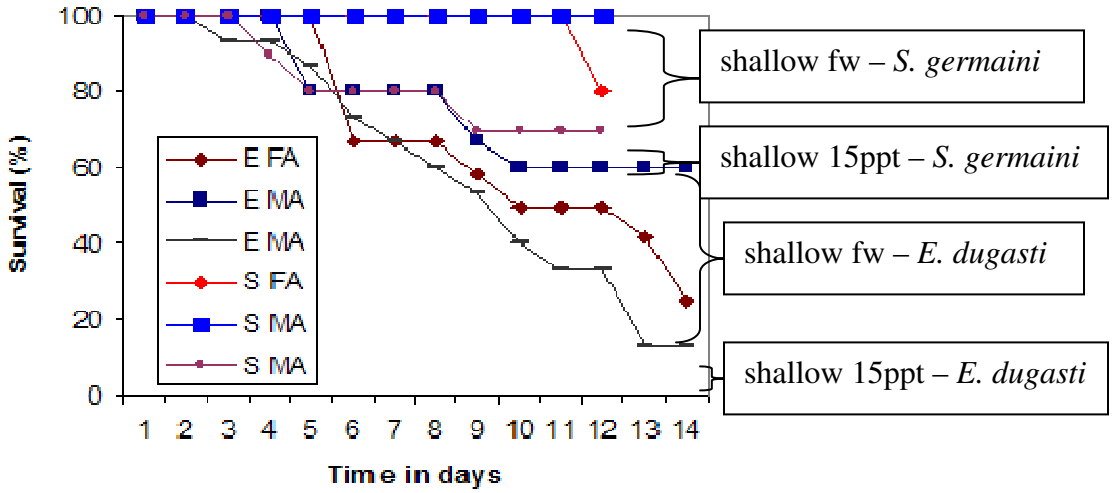


Fig. 3.24 Comparison of survival rates in adult *E. dugasti* (E) with *S. germaini* (S) in shallow fresh water and sea water at 15 ppt.

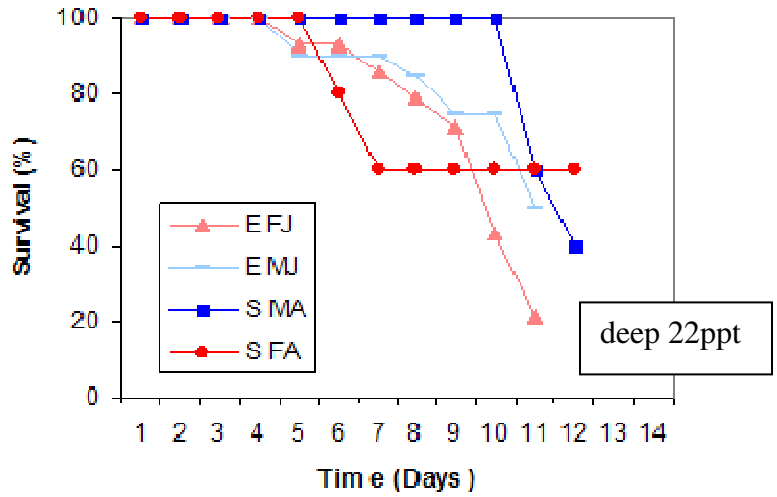


Fig. 3.25 Comparison of survival rates of juvenile *E. dugasti* (E) with adult *S. germaini* (S) in deep water at 22 ppt.

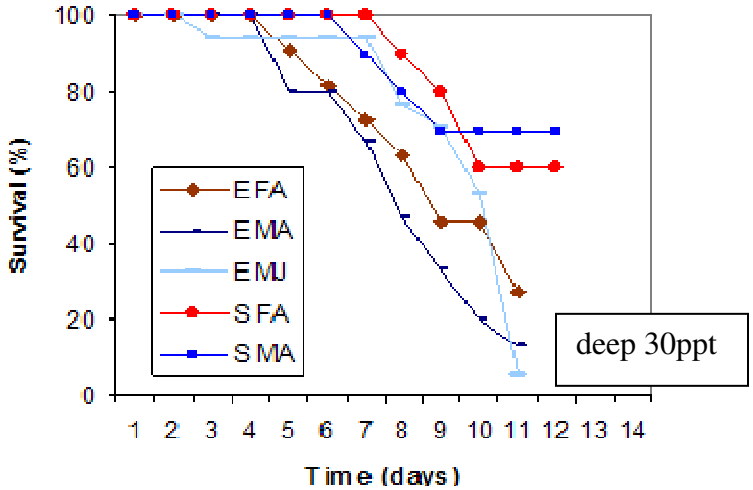


Fig. 3.26 Comparison of survival rates of adult and juvenile *E.dugasti* (E) with adult *S. germaini* (S) in deep water at 22 and 30 ppt.

3.5.3 *Comparison of survival within the Potamidae*

Survival of 'mini' juveniles of *L. chaiyaphumi* was 100 % in shallow 22 ppt, while 'mini' juvenile *P. smithianum* survived less well (Fig. 3.27). Juvenile *P. smithianum* also died after 11 days, while juvenile *L. chaiyaphumi* survived (Fig. 3.28).

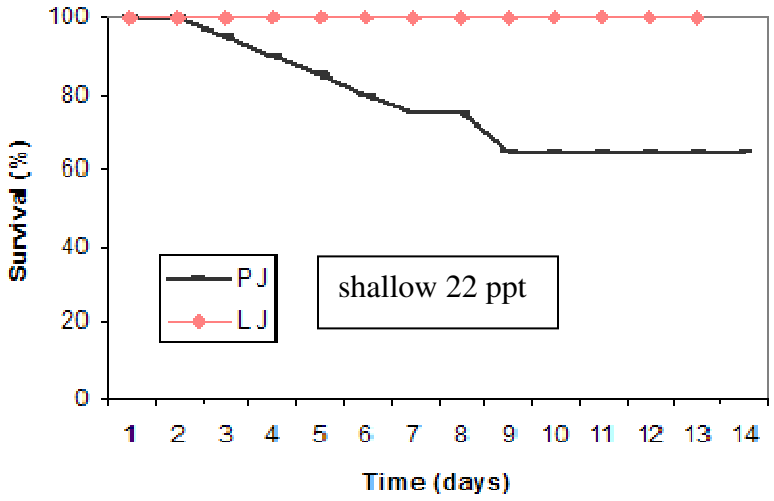


Fig. 3.27 Comparisons of survival rates of mini juvenile *P. smithianum* (P, n = 20) and *L. chaiyaphumi* (L, n = 10) in shallow water at 22 ppt

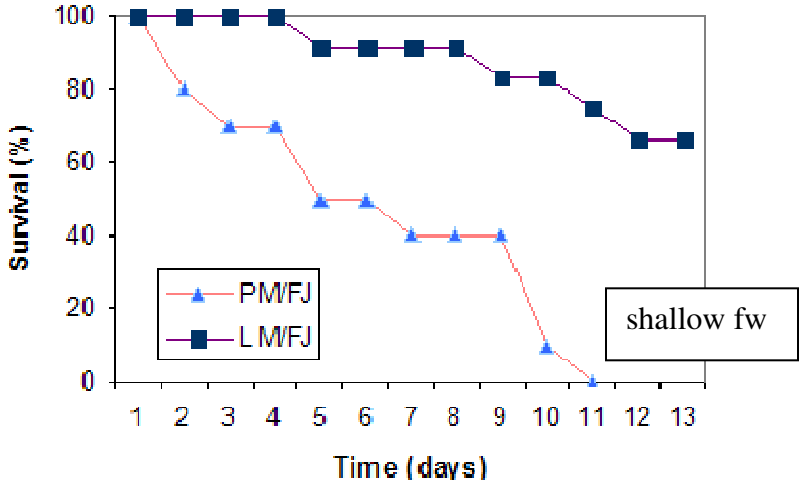


Fig. 3.28 Comparisons of juvenile *P. smithianum* (P, n = 10) and *L. chaiyaphumi* (L, n = 12) in shallow fresh water (0 ppt). J = juveniles, M/F = pooled males and females.

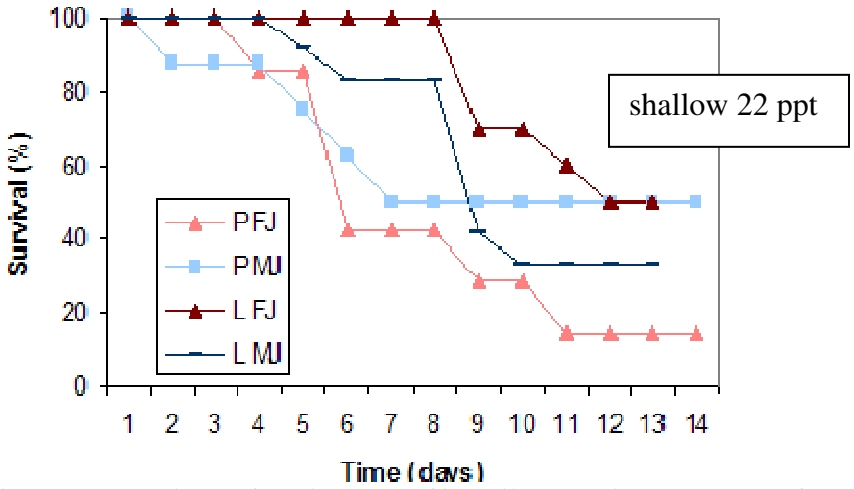


Fig. 3.29 Comparisons of survival rates of juvenile *P. smithianum* (P, n = 7 females and n = 8 males) and *L. chaiyaphumi* (L, n = 10 females, n = 12 males) in shallow water at 22 ppt.

Juvenile *L. chaiyaphumi* in shallow water at 22 ppt also survived better than *P. smithianum* for seven days, after which male juvenile *P. smithianum* survived better than male *L. chaiyaphumi*, at least until the tenth day (Fig. 3.29). All adult female *L. chaiyaphumi* in shallow 22 ppt sea water survived. The male and female adult *P. smithianum* in deep sea water at 28 ppt were the next best survivors while male *L. chaiyaphumi* and male and female *P. smithianum* subjected to 22 ppt had a survival rate of above 50% (Fig. 3.30).

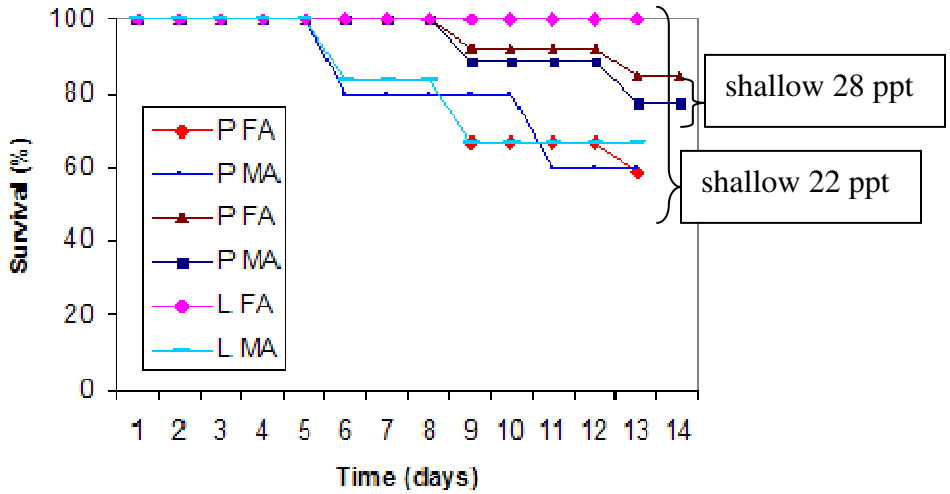


Fig. 3.30 Comparisons of survival rates of adult *P. smithianum* (P, n = 12 female and n = 5 male adults in water at 22 ppt and n = 13 for female and n = 9 big male adults in water at 28 ppt) and *L. chaiyaphumi* (L, n = 6 for each group) survival in shallow water at 22 ppt.

3.5.4 *Comparison between families in fresh water and sea water*

In shallow fresh water the probability of dying was decreased in *S. germaini* after 5 days in male adults (RR = 0.05) and after 9 days in male and female adults (RR = 0.03 and RR = 0.02, respectively) compared to sub-adult *E. dugasti.* Female adult *P. smithianum* had an increased probability of dying after 5 days in fresh water (RR = 20), but a reduced probability after 9 days (RR = 0.48) compared to *E. dugasti*. Juvenile male and female *P. smithianum* had an increased probability of dying (males RR = 60 on day 5 and 9, females RR = 5.20 on day 5 and R = 7.8 on day 9) compared to juvenile *E. dugasti* in fresh water. Female juvenile *L. chaiyaphumi* had an increased probability of dying (RR = 2.17 and 4.34 after 5 and 9 days, respectively) compared to female juvenile *E. dugasti* in fresh water, however, after 14 days, the probability was similar. Male juveniles of both species had a similar probability of dying over 14 days (Table F17).

The probability of dying in 30 ppt sea water increased in female and male adult *P. smithianum* after 5 days (RR = 4.4 and 2.5, respectively) compared to sub-adult *E. dugasti,* but was similar after 9 days. In 28 ppt the probability of dying over a 14 day period was constantly lower than that of sub-adult *E. dugasti* (RR = 0.12, 0.23 and 0.27 after 5, 9 and 14 days). The probability of dying in 30 ppt sea water was decreased after 5 and 9 days in adult *S. germaini* (females RR = 0.11 and 0.26, males RR = 0.05 and 0.42, respectively) compared to *E. dugasti.* The probability of dying in mini *L. chaiyaphumi* was similar to that of juvenile *E. dugasti* over a 14 day period (Table F18).

DISCUSSION

4. 1. Osmoregulatory ability

This study provides the first hemolymph osmolality values for any species of Asian freshwater crab and allows comparisons to other families of freshwater crabs from other continents (see Fig. 4.1. and Table 4.1).

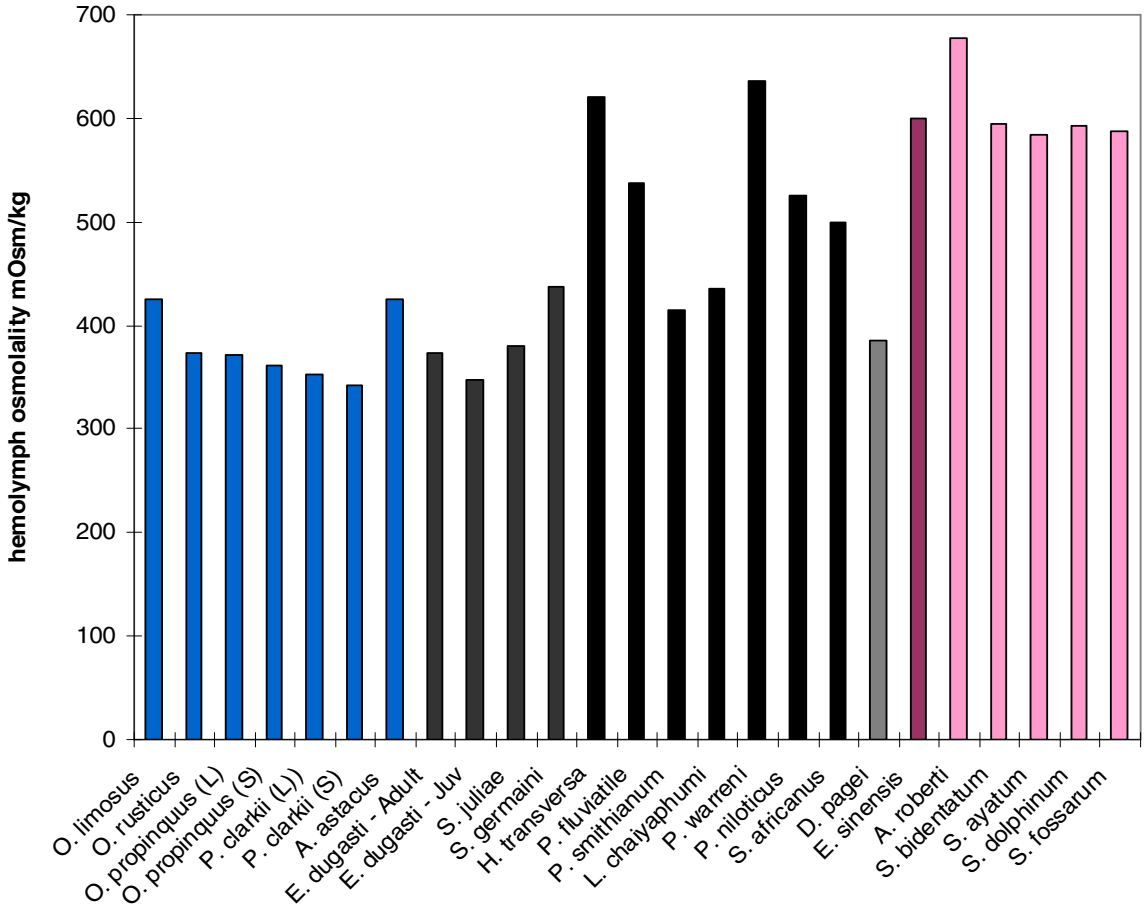


Fig. 4.1. Comparison of crayfish (blue columns), freshwater crab (grey and black columns), and grapsoid crab (pink columns) hemolymph osmolalities from different fresh water habitats. L = large adult specimen, S = small adult specimen.

Table 4.1 Comparison of hemolymph osmolalities of freshwater crabs, grapsoid crabs and crayfish in fresh water habitats. n/a when number of experimental animals and standard deviation were not available. L = large specimen, S = small specimen.

Species	**Superfamily**	**Family**	**Habitat type**	**Hemolymph in mOsm/kg ±SD**	**Source**
E. dugasti - Adult	Gecarcinucoidea	Parathelphusidae	FW	374 ± 32 (n=24)	This study
E. dugasti – Juv.	Gecarcinucoidea	Parathelphusidae	FW	348 ± 24 (n=20)	This study
S. juliae	Gecarcinucoidea	Parathelphusidae	FW	380 ± 14 (n=5)	Unpublished
S. germaini	Gecarcinucoidea	Parathelphusidae	FW	437 ± 32 (n=10)	This study
A. transversa	Gecarcinucoidea	Sundathelphusidae	FW	620 (n/a)	Mantel & Farmer, 1983
P. fluviatile	Potamoidea	Potamidae	FW	538 ± 97.5 (n=19)	Harris & Micallef, 1971
P. smithianum	Potamoidea	Potamidae	FW	415 ± 64 (n=20)	This study
L. chaiyaphumi	Potamoidea	Potamidae	FW	436 ± 39 (n=14)	This study
P. warreni	Potamoidea	Potamonautidae	FW	636 ± 80 (n=6)	Morris & Van Aardt, 1998
P. niloticus	Potamoidea	Potamonautidae	FW	525 (n/a)	Mantel & Farmer, 1983
S. aubryi	Potamoidea	Potamonautidae	FW	500 (n/a)	Mantel & Farmer, 1983
D. pagei	Portunoidea	Trichodactylidae	FW	386 ± 62 (n=12)	Onken & McNamara, 2002
A. roberti	Grapsoidea	Sesarmidae	FW	677.8 ± 32.9 (n=20)	Schubart & Diesel, 1998
S. bidentatum	Grapsoidea	Sesarmidae	FW – mountain streams	594 ± 23.6 (n=15)	Schubart & Diesel, 1999
S. ayatum	Grapsoidea	Sesarmidae	FW – mountain streams	584.9 ± 33.6 (n=10)	Schubart & Diesel, 1999
S. dolphinum	Grapsoidea	Sesarmidae	FW – mountain streams	593.6 ± 29.3 (n=19)	Schubart & Diesel, 1999
S. fossarum	Grapsoidea	Sesarmidae	FW – mountain streams	587.1 ± 18.4 (n=15)	Schubart & Diesel, 1999
S. windsor	Grapsoidea	Sesarmidae	FW - cave	530.1 ± 18.4 (n=15)	Schubart & Diesel, 1999
E. sinensis	Grapsoidea	Varunidae	FW / SW	FW: 600, SW: 900 to 1000 (n/a)	Onken et al. 2000, Mantel & Farmer, 1983
O. limosus	Astacoidea	Cambaridae	FW	425 (n/a)	Mantel & Farmer, 1983
A. leptodactylus	Astacoidea	Astacidae	FW	414.5 ± 6.8 (n=4)	Holdich et al. 1997
P. leniusculus	Astacoidea	Astacidae	FW	431± 4.7 (n=4)	Holdich et al. 1997
O. rusticus	Astacoidea	Cambaridae	FW	373 ± 32 (n=13)	Unpublished
O. propinquus (L)	Astacoidea	Cambaridae	FW	372 ± 63 (n=10)	Unpublished
O. propinquus (S)	Astacoidea	Cambaridae	FW	298 ± 51 (n=5)	Unpublished
P. clarkii (L)	Astacoidea	Cambaridae	FW	353 ± 88 (n=9)	Unpublished
P. clarkii (S)	Astacoidea	Cambaridae	FW	342 ± 44 (n=9)	Unpublished
A. pallipes	Astacoidea	Astacidae	FW	430 (n = 4, n/a)	Holdich et al. 1997
A. astacus	Astacoidea	Astacidae	FW	426 (n/a)	Mantel & Farmer, 1983

The hemolymph osmolality of Asian freshwater crabs in fresh water is in a range that is about one third that of sea water (398 mOsm/kg), with a mean value that is lower than that of the Old World European (540 mOsm/kg) and African freshwater crabs (553 mOsm/kg). Interestingly, the hemolymph of Asian freshwater crabs is similar to the mean hemolymph osmolality recorded for the New World trichodactylid freshwater crabs (386mOsm/kg) and for the freshwater crayfish (389 mOsm/kg). While little is known

about the origins of trichodactylid freshwater crabs, crayfish are a group that has a long evolutionary history of adaptation to fresh water. The hemolymph osmolality of New World sesarmids (*Armases* and *Sesarma*) in fresh water that have close relatives that breed in marine environments, is significantly higher than that of Asian freshwater crabs, which implies that these sesarmids invaded fresh water relatively recently (Schubart & Diesel, 1999). The hemolymph osmolality of the marine and euryhaline varunid *E. sinensis* falls into a similar range to that of the other grapsoids. The high hemolymph osmolality of the Australian parathelphusid freshwater crab *Australothelphusa transversa* (620 mOsm/kg) may reflect an adaptation to its seasonally arid environment.

All four freshwater crab species studied here (two parathelphusids and two potamids) were found to be able to survive in deep full strength sea water (30 ppt) for at least a week. In their natural habitat in fresh water, these crabs osmoregulate to maintain hemolymph osmolality hyperosmotic to the environmental water. These freshwater crabs (and perhaps other families of freshwater crabs) have been shown to be euryhaline freshwater animals (Kinne, 1963), despite the assertion by Shaw (1958b) that the potamonautid *Potamonautes niloticus* is oligohaline. The present work demonstrates that both parathelphusid and potamid freshwater crabs can survive for several days in brackish and full strength sea water, and that they normally osmoregulate in fresh water and dilute sea water by maintaining their hemolymph osmolality at hyperosmotic levels.

When exposed to water of increasing salinity, *E. dugasti* and *S. germaini*, the point at which the hemolymph became isosmotic (i.e., the salinity at which its osmolality is equal to that of the external medium) was approximated to be between 13 and 15 ppt (390 to 430 mOsm/kg). Interestingly, the hemolymph of freshwater crabs was never

observed to be perfectly isosmotic, and both parathelphusids kept their hemolymph osmolality slightly hyperosmotic to that of the water (13 or 15 ppt) they were in. When these parathelphusids were placed into water at a salinity that was isosmotic to their hemolymph, *S. germaini* and *E. dugasti* maintained their hemolymph hyperosmotic in water osmolalities up to 400 mOsm/kg and 390 mOsm/kg respectively (Figure 3.9b). It is not known whether the natural variation of the hemolymph within these crabs makes it difficult to determine whether crabs were isosmotic in 13 ppt or 15 ppt sea water. It is possible that freshwater crabs continue to osmoregulate even in water that is theoretically isosmotic to their hemolymph; i.e. they adjust their hemolymph osmolality so that it exceeds the isosmotic point and do not actually reach an isosmotic equilibrium. Such a strategy is seen in marine elasmobranchs (such as sharks) in full strength sea water and is thought to facilitate the slight entry of osmotic water into the body, so avoiding the necessity to drink sea water. This 'slightly hyperosmotic' strategy might explain why a range of hemolymph osmolalities was found in freshwater crabs in brackish water, rather than an exact steady state.

The hemolymph isosmotic point for the sesarmid *Armases roberti* that lives in fresh water permanently was at an osmolality of 867 mOsm/kg (Schubart & Diesel, 1998). The hemolymph in other species of the genus *Sesarma* that spend time in fresh water reached an isosmotic equilibrium with the external medium at around 24 ppt salinity (720 mOsm/kg) (Schubart & Diesel, 1999). The isosmotic points of different species of freshwater-living sesarmids (between 720 and 867 mOsm/kg) are clearly much higher than those found here for Asian freshwater crabs (390 to 400 mOsm/kg). Other indications of osmotic equilibrium in crabs in dilute media would be reflected in changes

in the basal metabolic rate and heart rate, because freshwater crabs maintaining isosmotic equilibrium in brackish water do not have to spend much energy on osmoregulation. This would be worth investigating.

When *E. dugasti* (Parathelphusidae), *P. smithianum* and *L. chaiyaphumi* (Potamidae) were in shallow water at 22 ppt their hemolymph was either hypo- or hyperosmotic, while that of *E. dugasti* and *S. germaini* (Parathelphusidae) in deep water at 22 ppt, was either hypo- or isosmotic to the water. Isosmotic levels of the hemolymph of *E. dugasti* were only reached after 13 days in water at 22 ppt, and water depth made no difference. The hemolymph osmolality of freshwater crabs studied here was found to vary widely, perhaps due to fluctuating water osmolality in the experimental tanks, and it could be that the hemolymph is tracking changes in environmental water osmolality. The gills of crabs are the main hyperosmoregulating tissues (Pequeux, 1995), but it is unclear whether hyposmoregulation is a result of the reversal of the same ion pumps or whether it occurs at a different location in the body such as the hindgut (Dall, 1967, cited in Schubart & Diesel, 1999). A salinity of 22 ppt might be the point at which some, but not all, freshwater crabs switch from hyper- to hyposmoregulation. Equally there could be an isosmotic range (13 to 15 ppt) where crabs stop osmoregulating and essentially osmoconform. Indeed, crabs in water at 22 ppt were observed to be livelier than those held in full strength sea water, while crabs maintained in fresh water were the liveliest and most vigorous of all.

The potamid *P. smithianum* had a survival rate of between 90 to 100% after 14 days in shallow water at 22 ppt, followed by 4 days in fresh water, and then 9 days in deep water at 25 ppt. Interestingly, the hemolymph osmolality of this potamid reverted

back to its original levels seen in freshwater some 3 days after returning to fresh water, indicating that adaptation to a change in external salinity is not instantaneous in freshwater crabs. A similar response has been reported in osmoregulating brachyuran crabs which took 48 hours to return to normal after exposure to salinity changes (Charmantier et al., 1998). It took up to 96 hours in the freshwater crayfish *Astacus leptodactylus* to return to normal (Susanto & Charmantier, 2000).

Esanthelphusa dugasti was the only species that did not hyperosmoregulate in water at 22 ppt which could indicate that this parathelphusid starts to switch from hyper- to hyposmoregulation at lower salinities than the other species. The fact that *E. dugasti* was hyposmotic for at least the first 9 days and only became isosmotic to the surrounding water after 13 days, could mean that its hyposmoregulatory ability starts to decrease after 9 to 13 days in a challenging external medium, and may explain its low survival rate after 14 days compared to the other species studied here. The smaller species size and the fact that sub-adults (rather than adults) were studied may also be contributing factors to the lowered survival rates seen in *E. dugasti*.

Potamon smithianum was hyposmotic in deep sea water at 25 ppt after 5 days, but hyperosmotic after the same amount of time in deep sea water at 28 ppt. It is not known whether *P. smithianum* in sea water at 28 ppt need more time to adapt so that it could hyposmoregulate, or whether after 5 days it had lost its hyposmoregulatory ability. The latter is thought to be more likely because in shallow sea water at 28 ppt the crabs were hyposmotic until the 9^{th} day but were found to be hyperosmotic after 13 days. In contrast, female sub-adults of *E. dugasti* in shallow water at 25 ppt were initially isosmotic, and became hyposmotic after 13 days when their hemolymph concentration increased from

between 610 and 682 to about 774 mOsm/kg. This might indicate that these animals hyposmoregulated whenever the osmolality of the external water reached about 700 mOsm/kg.

Esanthelphusa dugasti, S. germaini, and *L. chaiyaphumi* in shallow full strength sea water (30 ppt) all had hemolymph that was initially hyposmotic. Females of *E. dugasti* in deep full strength sea water (30 ppt) for 9 days had hemolymph that was still hyposmotic but that of males was either isosmotic or hyperosmotic after 9 days. When *E. dugasti* was in shallow sea water at 33 ppt its hemolymph was hyposmotic to the sea water, which might reflect its avoidance of the increased energy demands associated with osmoregulation in high salinities in deep water, brought about by a greater ease of avoidance of ion influx in shallow water when gills are only partially immersed. After 9 days in deep full strength sea water the hemolymph of *S. germaini* became almost isosmotic to full strength sea water after previously osmoregulating at hyposmotic hemolymph levels for at least 8 days. When *L. chaiyaphumi* 'mini' juveniles were in sea water at 30 ppt (850 mOsm/kg) for 12 days they first hyporegulated and then osmosconformed, which resulted in their hemolymph osmolality matching that of sea water.

The ability of freshwater crabs to hyposmoregulate in sea water appears to be typical of terrestrial and semi-terrestrial freshwater crustaceans, such as the sesarmids *Armases miersii* and *Sesarma curacaoense.* Anger (2001) explained this ability in sesarmids as a compensation to enhanced hemolymph osmolality caused by desiccation during terrestrial activity. The African potamonautid *P. warreni* was observed to osmoregulate in "lower salinities" but failed to osmoregulate after one week in high

salinity (80% SW, ~ 22.8 ppt) when Cl^- ion-regulation failed, followed by the failure of its Na^+ and Mg^{2+} ion-regulation after two weeks. When *P. warreni* was in 40% SW (~ 11.4 ppt) its Na^+ ion-regulation and therefore its osmoregulation was maintained successfully for three weeks (Morris & van Aardt, 1998). The failure of individual ion pumps over time could explain the increases in hemolymph osmolality observed in each of the four species of freshwater crabs studied here, particularly when in salinities over 22 ppt. Five Jamaican sesarmid species (genus *Sesarma*) studied by Schubart & Diesel (1999) showed a similar osmoregulatory pattern, in which crabs in salinities from 0 up to 8 ppt (0 up to 250 mOsm/kg) responded with a distinct increase in internal osmotic concentration, while crabs in salinities from 8 to 24 ppt (250 to 720 mOsm/kg) reached equilibrium with the external medium at around 24 ppt salinity. Above this salinity, crabs showed a rapid increase in their internal osmolality whereby their hemolymph eventually conformed to the external medium (Schubart & Diesel, 1999).

Parathelphusidae: *Esanthelphusa dugasti* and *Sayamia germaini.* In general salinity had a significant effect on hemolymph osmolality in *E. dugasti* and *S. germaini*, but statistical support was low. Salinity was expected to be a significant factor for both species, especially for *S. germaini* because the salinities chosen were at least 7 ppt apart (control, 15, 22 and 30 ppt). Water depth effected hemolymph osmolality in *E. dugasti* but again statistical support was low. Juvenile *E. dugasti* had a significantly lower hemolymph osmolality than sub-adults after 13 days in high salinity (30 ppt), but statistical support was low. It would appear that small increases of the salinity of the environmental water (up to 7 ppt) do not greatly challenge the osmoregulatory

mechanisms of these Asian freshwater crabs judging by the fact that hemolymph osmolality did not change significantly (Table 3.2).

Potamidae: *Potamon smithianum* and *Larnaudia chaiyaphumi.* The salinity of the environmental water was the most significant factor in altering the hemolymph osmolality of both potamids. In general salinities that were more than 6 ppt apart resulted in a significant change in hemolymph osmolality of freshwater crabs in sea water. Neither gender nor age had a significant effect on the hemolymph osmolality of *P. smithianum*. In crayfish, however, a significant difference in hemolymph osmolality was found between adult (420 mOsm/kg) and hatchling (290 mOsm/kg) crayfish, *Astacus leptodactylus* (Susanto & Charmantier, 2000).

Interpretation of factors

The influence of water depth at high salinity on the hemolymph osmolality of freshwater crabs needs to be further investigated because of the potential for salinity to vary due to desiccation at high air temperatures when out of water. It is possible that when crabs are in deep full strength sea water ions are more successfully pumped out across their gills when submerged, than those in shallow full strength sea water when their ion pumps are compromised. Depending on species, freshwater crabs spend their entire life cycle either fully submerged in fresh water streams, rivers, or lakes, or they divide their time between life in water, life on land (semi-terrestrial), and life in humid burrows (Ng, 1988; Cumberlidge, 1999). Many species of parathelphusids live in rice fields (i.e. semi-terrestrial shallow water) while many species of potamids live in highly oxygenated deep water environments such as land waterfalls and rivers (Table A2). The ability of freshwater crabs to survive for between one and two weeks when fully

submerged in sea water is an indirect measure of their potential to be carried across short-distance saltwater barriers perhaps by clinging to floating vegetation and driftwood. Indeed, parathelphusids and potamids were shown here to survive successfully for 9 and 13 days, respectfully, in shallow water under high salinity conditions, mimicking the harsh conditions experienced by a freshwater crab clinging to floating vegetation in the ocean. Such events would also expose crabs to emersion in air, splashing by seawater, and high temperatures. Emersion itself does not cause a significant increase in hemolymph osmolality, as was demonstrated in the South African potamonautid freshwater crab *Potamonautes warreni* (Morris & van Aardt, 1998). However, Lutz (1969) demonstrated an increase in the percentage of ions in the plasma after 24 hours desiccation in air in *Sudanonautes aubryi* (formerly identified as *S. africanus africanus,* see Cumberlidge, 1999) which Lutz attributed to the concentrating effect on the hemolymph of increased evaporation. Mild desiccation results in water loss from the extracellular fluid (hemolymph), but largely protects the intracellular fluid within the cells from water loss. Although mild desiccation leads to a rise in the concentration of most solutes except sodium, more severe desiccation can affect cells and can lead to the breakdown of the sodium control mechanism itself (Lutz, 1969).

In general, gender did not have an important effect on hemolymph osmolality in either parathelphusid or potamid freshwater crabs. A gender difference in hemolymph osmolalities might have been a reasonable expectation in Indian gecarcinucids, because significantly higher levels of free amino acids in the hemolymph of males compared to females have been reported (Padmanabhanaidu & Ramamurthy, 1961). The protein content of individual tissues of the Indian gecarcinucid *Barythelphusa guerini* was found

to depend on both gender and the size of the animal (Venkatachari & Ambore, 1973). Those authors concluded that sex and the age (size) should be kept constant in osmoregulatory studies because initial levels of nitrogen pools in the tissues play a role in isosmotic intracellular regulation (Florkin & Schoffenfiels, 1965). In contrast, plasma ions levels did not correlate with gender in the African potamonautid freshwater crab *S. aubryi* (Lutz, 1969) or in the sesarmid *A. roberti* (Schubart & Diesel, 1998). Although the chemical composition of the hemolymph was reported to be different between *Parathelphusa* (Parathelphusidae) and *Sudanonautes* (Potamonautidae), it cannot be used for direct comparison here because neither study measured hemolymph osmolality (Padmanabhanaidu & Ramamurthy, 1961, Lutz, 1969).

Age was found to be an important factor in explaining the survival ability of freshwater crabs subjected to different salinities, but not as a factor to determine hemolymph osmolality. Age (as judged by body weight), was found to be important in Indian gecarcinucids (Padmanabhanaidu & Ramamurthy, 1961) with respect to hemolymph chloride concentration where maximal concentrations were found in large adult male (40 g) and female (35 g) crabs. In the present study hemolymph osmolality was only found to be different in specimens of different ages in the parathelphusid *E. dugasti,* but was not significantly different in the potamids.

The osmoregulatory ability of freshwater crabs belonging to different families is particularly interesting when addressing important questions regarding their distribution and dispersal abilities. The present findings that adult, sub-adult, juvenile, and 'mini' juvenile freshwater crabs can survive for up to two weeks in full-strength seawater was unexpected because freshwater crabs are never found naturally in either brackish water,

in estuaries, or in the ocean. The potamid *Johora tiomanensis tiomanensis* has often been collected very near the sea on the Malaysian Island of Pulau Tioman, where many of the streams, rivers and waterfalls flow through steep and hilly terrain and often are still flowing rapidly when they meet the sea. *Johora* is more habitat specific than altitude specific, so its presence very close to the sea is not necessarily surprising (Ng, 1988). The parathelphusid *Sayamia germaini* is reported to be sympatric with *Siamthelphusa improvisa* (Ng, 1988, as *Somanniathelphusa germaini*) and *Siamthelphusa improvisa* "has been found close to the sea and can almost certainly tolerate tidal changes and small changes in salinity". It is not known whether it is the inability of the eggs of freshwater crabs to survive in saltwater that is the major limiting factor affecting the distributional range of a species. The inability of these presumably more vulnerable stages to survive in brackish or full strength sea water might impose limits on a species range expanding into salt water habitat. If this were found to be the case, then it would explain why freshwater crabs are strictly confined to freshwater environments. Freshwater crabs possess direct development and lack the vulnerable planktonic developmental stages seen in marine crabs. In marine homarid lobsters salinity tolerance of adults and larvae is different (Charmantier et al., 2001) which explains the migratory movements of some species of lobster in and out of subtidal and estuarine environments, especially adult females. In freshwater crabs, the closest stage to the larval stage is the hatchling crab, represented in the present study by 'mini' juvenile stages. These 'mini' crabs were shown here to have survival abilities that were equal to, or even superior to those of adult crabs.

Species comparison

It is interesting that in fresh water the natural environment for freshwater crabs *E. dugasti* and *P. smithianum* always have significantly different hemolymph osmolalities from each other. The parathelphusid *E. dugasti* always had the lower osmolality (374 ± 32 mOsm/kg in shallow water), while that of the potamid *P. smithianum* was always higher (415 ± 64 mOsm/kg in shallow water) (Tables 4.1). After 5 or 9 days in 30 ppt sea water the probability of dying in *S. germaini, P. smithianum* and *L. chaiyaphumi* was always lower than that of sub-adult *E. dugasti*, except for adult *P. smithianum* after 5 days. The higher probability of dying in *E. dugasti* might be associated with its smaller size as a sub-adult compared to adults rather than with salinity stress itself.

Schubart and Diesel (1998) argued that a comparatively high osmotic hemolymph (hyperosmotic) concentration of sesarmids that live in fresh water (e.g. *A. roberti,* 680 mOsm/kg, Table 4.1), and their ability to hyposmoregulate in salt water reflect a fairly recent invasion of the fresh water habitat from the marine supralittoral. The hemolymph osmolality of the true freshwater crabs studied here is typically in a significantly lower range of values compared to that of marine sesarmids that live in fresh water. Depending on species, freshwater crabs have either a low hemolymph osmolality that is similar to that of crayfish (*E. dugasti*), or one that is a slightly higher than crayfish (e.g., *S. germaini, P. smithianum,* and *L. chaiyaphumi*, Table 4.1, Fig. 4.1). The higher hemolymph osmolality levels of these latter three species of freshwater crabs when compared to crayfish could mean that these freshwater crabs are more recent invaders of fresh water than are crayfish, while the higher hemolymph osmolality levels in sesarmids indicate that these sesarmids have been adapting to fresh water environments for a much

shorter time compared to freshwater crabs. In sea water at 22 ppt the parathelphusid *E. dugasti* had a significantly lower hemolymph osmolality on day 9 compared to the other species, but had the highest hemolymph osmolality on day 13. Although it was not significantly higher than *P. smithianum*, the hemolymph osmolality of both species were significantly higher than that of *L. chaiyaphumi* (Table 3.22). The potamid *P. smithianum* had the best hyposmoregulatory ability judging by its consistently low levels of hemolymph osmolality in full strength sea water (30 ppt), although this difference between species disappeared after 13 days in sea water (Table 3.23).

4.2 Survival ability

All four freshwater crab species studied here can cope for up to two weeks with experimental ambient salinities ranging from fresh water to full strength sea water. These crabs either hyper- or hyporegulate and are not necessarily isosmotic in higher salinities. Other species of parathelphusids from Thailand such as *Siamthelphusa improvisa* are not aggressive animals and can be kept together in relatively large numbers without limb loss or damage, unless they are moulting (Ng, 1988). However, potamids are more aggressive, and species from Peninsular Malaysia and Singapore (e.g., *Johora johorensis johorensis, Johora johorensis murphyi, Stoliczia stoliczkana stoliczkana, Stoliczia chaseni*, *Stoliczia tweedei*) cannot be maintained together in captivity without conflict (Ng, 1988). The exact cause of death of freshwater crabs in high salinities cannot be determined here because it could be due either to their ultimate failure to sustainably osmoregulate in the long-term (Table 4.2), or to sampling stress and a general weakening resulting from limb loss (and cannibalism in the case of *E. dugasti*). Multiple autotomy of limbs triggers the

onset of proecdysis and a post autotomy intermolt cycle that is significantly shorter than normal, at least in marine fiddler crabs (*Uca)* (Hopkins, 1982), but it is not known whether this is also the case for freshwater crabs. High temperature, light regime, and the presence of other crabs, are all factors known to hinder larger species from molting, but small species of marine fiddler crabs (e.g., female *Uca* and male *Ocypode*) can molt under these conditions (Weis, 1976) and it is possible that small species of freshwater crabs (*E. dugasti*) can also molt under these conditions.

Table 4.2 Hemolymph osmolality of recently deceased freshwater crabs

					hemolymph		water	hemolymph
salinity	Water depth	day	gender	age	Osmolality mOsm/kg	color	Osmolality mOsm/kg	Group osmolality
E. dugasti								
fw	shallow	9	F	sub-adult	460	colorless	50	365
7 ppt	shallow	-	M	sub-adult	614	milky	222	399
22 ppt	shallow	-	F	sub-adult	828	milky	756	627
22 ppt	shallow	14	F	juvenile	722	orange	697	617
28 ppt	shallow	-	F	sub-adult	739	colorless	753	664
30 ppt	shallow	13	F	juvenile	1034	yellow	827	766
30 ppt	shallow	13	M	juvenile	925	yellow	968	767
33 ppt	shallow	-	F	sub-adult	888	milky	847	987
S. germaini								
15 ppt	shallow	5	M	adult	562	milky	380	510
30 ppt	deep	9	F	adult	1109	orange	805	760
P. smithianum								
30 ppt		5	M	adult	888	milky	750	779

When the North American freshwater crayfish *Orconectes virilis* was held experimentally in high salinity water the incidence of molting increased and the individuals that molted died sooner that those that did not molt (Kendall & Schwartz, 1964). The lower percent survival of the parathelphusid *E. dugasti* in fresh water found here compared to the other freshwater crab species in fresh water could be explained by the above mentioned combination of factors, because those animals that survived until

the very last day of the experiment were those that were still in good condition and that had not suffered the loss of limbs or agility.

Despite the high ambient temperatures, survival was reasonably high under the experimental conditions in the present study. Only four out of the 63 experimental groups here had no surviving animals at the end of the experiment. As might be expected, full strength sea water was the most stressful to these crabs. In some instances where mortality was high and salinity stress was not a factor (i.e., low survival rates in crabs in freshwater), the initial poor condition of the animals is suspected (Fig. 3.16a, Table 4.2). Fifty-nine experimental groups comprising two species of parathelphusids and two species of potamids always had some survival over the time of the experiment for 9 to 14 days, even in full strength sea water (30 ppt).

The African potamonautid *P. niloticus* can survive for up to 3 weeks in 75% SW (21.4 ppt), but only survived for four days in 100% SW (Shaw, 1958b). This contrasts with the European potamid *Potamon*, which survived for at least a month in full strength sea water, although the percentage of animals that survived was not reported (Shaw 1958b). The admittedly small sample of Asian freshwater crabs from this study (Table A1) indicates that not all species or families of freshwater crabs are similarly intolerant of sea water.

Esanthelphusa dugasti. Strong salinities and shallow water have the greatest influence on the survival of *E. dugasti* after 5 days, whereas salinity regime and age of the crabs (rather than water depth) was more important for survival over longer exposures (9 to 13 days) to high salinities. However, the water depth had an important influence on survival only during the first few days, after which crabs became adapted to their new

conditions, and it was the salinity regime and / or their age that influenced their survival after 9 days. It is always possible that survival due to the age of crabs could be an artifact of the experimental protocol, because juvenile crabs were bled once (after 13 days), while adults were bled three times (on days 5, 9 and 13).

Sayamia germaini. All groups held in fresh water for 11 days had 100% survival, indicating that the long-term health of these individuals was not altered by the earlier stressful transport that initially killed 50 % of the animals collected. After 5 days, all experimental groups had 100% survival (except for the male adults subjected to shallow 15 ppt water). Male crabs kept in shallow water did not survive initially and the survival of males also decreased over time, indicating that male adult *S. germaini* might differ in their osmoregulatory ability from females. Salinity and survival have an inverse relationship: an increase in salinity resultes in a decrease in the survival rates.

Potamon smithianum. Initially salinity was the only significant factor that affected survival. However, after 5 days, survival was better in 28 ppt than in 25 ppt sea water, therefore age was a more important factor than salinity.

Larnaudia chaiyaphumi. All groups of showed 100% survival after 5 days in all salinities. After between 9 and 13 days in high salinities, the 'mini' juveniles had the highest survival rate, even in full strength sea water (30 ppt), but juveniles did not survive as well as adults. This could mean that adults take longer than juveniles to develop their osmoregulatory response, but once adapted, adults have more energy stores than do juveniles to support sustained osmoregulation.

Species comparison. In general, salinity and age can be used to predict survival. Age was an important predictor of survival for potamids after 9 days in high salinity

water, but salinity was the main factor for the parathelphusid *E. dugasti*. Age was a significant factor in the survival of *E. dugasti*, *P. smithianum* and *L. chaiyaphumi* and presumably *S. germaini*. The survival rate of adult *E. dugasti* or *L. chaiyaphumi* in high salinity water decreased after 9 days, while adult *P. smithianum* survived well throughout this period. Juvenile potamids (*P. smithianum*) survived less well in high salinity water than did adults, but juveniles of *L. chaiyaphumi* had a higher probability of surviving than adults, and 'mini' juveniles had the highest probability of survival of all.

Young freshwater crabs molt more often than adult freshwater crabs and are assumed to use more energy for osmoregulation during the soft-shell phase. In this context it is perhaps surprising that the mini juvenile potamids survived as well, as they did, considering that the surface-to-volume ratio (osmosis and diffusion surfaces) is greater for smaller animals. Unfortunately the number of mini juveniles was not high enough to also subject *P. smithianum* to the same range of salinities as possible for *L. chaiyaphumi*. No comparative survival data for freshwater crabs are available from published studies that specifically controlled for the effects of age, gender and water depth.

The marine-breeding varunid *E. sinensis* is found in freshwater, has regulatory capabilities and euryhaline abilities that continually increase throughout juvenile growth. By adulthood it has gradually attained the ability to cope equally well with fresh water and sea water. Cieluch et al. (2007) found an ontogenetic increase in osmoregulatory capacity of *E. sinensis* zoea larvae which hyperosmoregulated in dilute media, but osmoconformed in sea water and at higher salinities (>32.2 ppt). *Eriocheir sinensis* megalopae and stage I-II juveniles hyperosmoregulated at low salinities and

hyposmoregulated at >32.2 ppt. Survival in sea water at ca. 10 to 32 ppt was generally high (90 to 100%), while complete mortality occurred in all zoeal stages (except zoea I) at 0.16 to 5.3 ppt. The mini juveniles of the potamid *L. chaiyaphumi* had 100% survival after 13 days in 22 ppt, but only 90% in fresh water and 70% in 30 ppt.

In this study, the greater survival of 'mini' juveniles of *L. chaiyaphumi*, compared to juveniles and adults may have been due to the lack of hemolymph withdrawal because of their small size. An alternative possibility could be that osmoregulation in juvenile freshwater crabs is different from that in adults, especially in potamids. It was found that in *E. sinensis* megalopa and juvenile crab stage I, ionocytes and immunolabeled Na^+/K^+-ATPase were located in the filaments of the most posterior gills, while no immuno-localization occurred in the anterior gills (Cieluch et al., 2007). There might be a close relationship between the ontogeny of osmoregulation and the expression of ATPase within the transporting epithelia of the branchial chamber. Cieluch et al. (2007) concluded that the adult pattern of osmoregulation developed in *E. sinensis* through two molts, from a moderately hyper-isoosmoregulation zoeal phase to the moderately hyper-hyposmoregulating first juvenile crab stage. The osmoregulatory capacity and salinity tolerance increased in subsequent juvenile instars and the regulatory abilities continued to increase gradually throughout the subsequent juvenile phases, until it fully reached an osmotic concentration of >600mOsm/kg in adults in fresh water (De Leersnyder, 1967). The species-specific adult pattern of hyper-hyposmoregulation appeared for the first time in the megalopa stage, e.g., in *E. sinensis* (Cieluch et al., 2007), *Armases miersii* (Charmantier et al., 1998), *Sesarma curacaoense* (Anger & Charmantier, 2000),

Chasmagnathus granulata (Charmantier et al. 2002), *Carcinus maenas* (Cieluch et al., 2004), and *Uca subcylindrica* (Rabalais & Cameron, 1985 cited in Cieluch et al., 2007).

There seems to be a similar timing of the ontogenetic changes in the species belonging to four different brachyuran families (Varunidae, Sesarmidae, Portunidae, Ocypodidae) that are tolerant of great salinity fluctuations. This ontogenetic pattern of osmoregulation may be typical of euryhaline decapods in general including the freshwater crabs, even though they have direct development (i.e., freshwater crabs pass through the megalopa stage inside the egg case). Some sesarmid crabs that live in fresh water have abbreviated development, which is also a different strategy than seen in marine crabs and freshwater crabs.

4.3. *Biogeographical implications*

This study examined the osmoregulatory ability of Asian freshwater crabs from two different families in order to determine whether these animals could survive short periods of exposure to sea water, thereby making it possible for them to travel short distances across oceanic barriers to nearby islands.

The ability to osmoregulate for short periods in dilute or full strength sea water in laboratory conditions does not precisely reproduce the natural environmental conditions, where crabs face might also face threats from competitors and predators that are well adapted to brackish or marine waters. For this reason, survival in high salinity water in laboratory conditions does not mean that the species could competitively survive for long in a natural marine environment. For example, experimental animals exposed to full

strength sea water for extended periods tended to become less active and/or less robust as judged by their resistance during hemolymph extraction.

Juveniles and adults were of particular interest in this study in the determination of whether different life stages had a different ability to survive in sea water and thereby able to cross a salinity barrier. The results of this study show that it is reasonable to believe that true freshwater crabs from 'mini' to adult age might have survived for up to two weeks, which would be long enough to be carried short distances for example between the mainland and nearby islands.

A systematic study of potamid freshwater crabs of the genus *Geothelphusa* found that specimens identified as *G. lanyu* and *G. lutao*, from Lanyu and Lyudao Islands respectively off the southern coast of Taiwan island actually belong to *G. tawu* from the main island of Taiwan (Shih et al., 2004). This is an interesting finding because offshore volcanic islands of Lanyu and Lyudao were never connected to Taiwan island in the past and are separated from Taiwan proper by deep oceanic troughs of 2,500 m and 3,000 m, respectively. This means that vicariance explanations of how the same species came to be distributed on the main island and on two offshore islands cannot be invoked. Similarly, past connections formed by a land bridge exposed by a drop in sea level in the past cannot explain the presence of the same species on the mainland and on these islands because even a sea level decrease of 120 m would not have connected these islands either to each other or to the main island (Shih et al., 2004). The last volcanic activity on these islands is estimated to have been 0.54 mya (Lanyu Island) and 1.4 mya (Lyudao Island) (Chen, 1990, cited in Shi et al., 2004). The presence of *G. tawu* on these islands, indicates

that populations must have colonized the islands after the last volcanic explosions and presumably must have crossed the sea to do so.

Shih et al. (2004) proposed three mechanisms to explain how freshwater potamids which are supposedly intolerant of salt water, could have reached these islands across a salt water barrier. One mechanism could be violent typhoons with very heavy rainfall and flooding rivers might have carried freshwater crabs from mountain streams into estuaries and then to offshore islands. Another mechanism could be mats of floating vegetation on which the crabs might have been stranded could have carried crabs to these islands with a decreased chance of contact with sea water. Semi-terrestrial habits (air breathing and resistance to desiccation) and heavy rainfall could have also increased the chances of surviving passage to a distant island on a vast mat of floating vegetations. A third mechanism that does not assume sea water tolerance is the accidental transport of crabs by birds, e.g. a bird attack on a female brooding crab that would result in hatchlings being released from the brood pouch and scattered onto the body and feathers of the bird. Shih et al. (2004) admit that this would be a rare event. Alternatively, humans traveling between the islands in canoes might have brought freshwater crabs either by chance within their drinking water, or on purpose as food items. It is known that freshwater crabs are eaten by aborigines, and it is possible that captive animals (adults or hatchlings) could have been accidentally released on these islands.

Passive transport on a mat of uprooted vegetation (flotsam) as a means of dispersal has been reported to carry green iguanas from Guadeloupe island in the Caribbean to Anguilla, following hurricane devastation (Censky et al., 1998). This same method has been used to explain how Indian rice frogs (*Rana limnocharis*) reached

Lanyu and Lyudao Islands from Taiwan proper (Toda et al., 1998, cited in Shih et al., 2004).

4.4 *Conclusion*

All four species of freshwater crabs studied here were found to be able to osmoregulate in a range of salinities, and all were able to surviving in salinities of up to 30 ppt for at least 5 days, and often much longer. Salinity seemed to be the most important factor determining survival, followed by age, whereby recently hatched crabs or adults had higher survival rates than did juveniles. Despite low survival rates in high salinity after 14 days, only a few survivors would be needed for successful colonization after short distance crossings between continents and nearby islands.

Improved phylogenetic trees in combination with molecular dating may make it possible in the future to examine these hypotheses of specific short distance crossings through saltwater barriers in more detail. Further systematic studies are needed for the South East Asian freshwater crab fauna. Presently, more African than Asian freshwater crabs have been sequenced and while the phylogeny and higher taxonomy of Afrotropical freshwater crabs has just been revised (Cumberlidge et al., 2007), the Asian freshwater crabs are still in need of a major taxonomical revision.

LITERATURE CITED

Anger, K. & G. Charmantier, 2000. Ontogeny of osmoregulation and salinity tolerance in a mangrove crab, *Sesarma curacaoense* (Decapoda, Grapsidae). *Journal of Experimental. Marine Biology and Ecology,* **251**: 265–274.

Bachmeyer, F. & G. Pretzmann, 1971. Krebsreste aus den Altpliozaenen Suesswasserablagerungen des Eichkogels bei Moedling, Niederoesterreich. *Annalen des Naturhistorischen Museums in Wien,* **75**: 283–291.

Banarescu, P., 1990. *Zoogeography of freshwaters – Vol. 1 General Distribution and Dispersal of Freshwater Animals*. Aula Verlag, Wiesbaden. 511 pp.

Boltt, G. & J. Heeg, 1975. The osmoregulatory ability of three Grapsoid crab species in relation to their penetration of an estuarine system. *Zoologica Africana,* **10**: 167–182.

Bossuyt, F., M. Meegaskumbura, N. Beenaerts, D.J. Gowe, R. Pethiyagoda, K. Roelants & et al. 2004. Local endemism within the Western Ghats-Sri Lanka Biodiversity Hotspot. *Science,* **306**: 479–481.

Bott, R., 1955. Die Suesswasserkrabben von Afrika (Crust., Decap.) und ihre Stammesgeschichte. *Annales du Musee Royal du Congo Belge C. Zool.*, Serie III, III 1: 209–352.

Bott, R., 1969. Praeadaptation, Evolution and Besiedlungsgeschichte der Suesswasserkrabben der Erde. *Natur und Museum* 99, 266-275.

Bott, R., 1970. Die Suesswasserkrabben von Europa, Asien, Australien und ihre Stammesgeschichte. *Abhandlungen Senckenbergia Naturforschenden Gesellschaft, Frankfurt am Main,* **518**:

Bott, R., 1972. Stammesgeschichte und geographische Verbreitung der Suesswasserkrabben. *Natur und Museum* 102, 63-77.

Brandis, D., 2002. On the taxonomic status and biogeography of the Isolapotamidae Bott 1970 (Decapoda, Brachyura). *Journal of Natural History*, **36**: 1291–1339.

Censky, E.J., K. Hodge, & J. Dudley, 1998. Overwater dispersal of lizards due to hurricanes. *Nature,* **395**: 556.

Chakrabarty, P., 2004. Cichlid Biogeography: Comment and Review. *Fish and Fisheries,* **5**: 97-119.

Charmantier, G., M. Charmantier-Daures, & K. Anger, 1998. Ontogeny of osmoregulation in the grapsid crab *Armases miersii* (Crustacea: Decapoda). *Marine Ecology Progress Series,* **164**: 285–292.

Charmantier, G., C. Haond, J.H. Lignot, & M. Charmantier-Daures, 2001. Ecophysiological adaptation to salinity throughout a life cycle: A review in Homarid Lobsters. *The Journal of Experimental Biology,* **204**: 967–977.

Charmantier, G., L. Gimenez, M. Charmantier-Daures, & K. Anger, 2002. Ontogeny of osmoregulation, physiological plasticity and larval export strategy in the grapsid crab *Chasmagnathus granulate* (Crustacea, Decapoda). *Marine Ecology Progress Series,* **229**: 185–194.

Chuensri, C., 1974. Freshwater crabs of Thailand. *Fisheries Research Bulletin*, Kasetsart University, 7: 1–47.

Cieluch, U., K. Anger, F. Aujoulat, F. Buchholz, M. Charmantier-Daures, & G. Charmantier, 2004. Ontogeny of osmoregulatory structures and functions in the green crab, *Carcinus maenas* (Crustacea, Decapoda). *Journal of Experimental Biology*, **207**: 325–336.

Cieluch, U., K. Anger, M. Charmantier-Daures & G. Charmantier, 2007. Osmoregulation and immunolocalization of Na^+/K^+ - ATPase during the ontogeny of the mitten crab *Eriocheir sinensis* (Decapoda, Grapsoidea). *Marine Ecology Progress Series,* **329**: 169–178.

Cumberlidge, N., 1999. *The Freshwater Crabs of West Africa – Family Potamonautidae.* Institut de recherche pour le developpement, Paris. 382 pp.

Cumberlidge, N., S.R. Daniels, & R.v. Sternberg, 2007. A revision of the higher taxonomy of the Afrotropical freshwater crabs (Decapoda: Brachyura) with a discussion of their biogeography. *Biological Journal of the Linnean Society* (in press).

Daniels, S.R., N. Cumberlidge, M. Perez-Losada, S.A.E. Marijnissen, & K.A. Crandall, 2006. Evolution of Afrotropical Freshwater Crab lineages obscured by Morphological convergence. *Molecular Phylogenetics and Evolution,* **40**: 227-235.

Dai, A.-Y., G.X. Chen, & Y.Z. Song, 1979. On new species of freshwater crabs harbouring metacercariae of lung flukes. *Acta Zootaxonomica Sinica,* **4** (2): 122–131.

De Leersnyder, M., 1967. Le milieu interieur d'*Eriocheir sinensis* H. Milne Edwards et ses variations. I. Etude dans le mileu naturel. *Cahiers de biologie marine,* **8**: 195–218.

Farias, I.P., G. Orti, & A. Meyer, 2000. Total evidence: Molecules, Morphology, and the Phylogenetics of Cichlid Fishes. *Journal of Experimental Zoology (MOL DEV EVOL),* **288**: 76–92.

Flik, G. & C. Haond, 2000. Na^+ and Ca^{2+} pumps in the gills, epipodites and branchiostegites of the European lobster *Homarus gammarus*: Effects of dilute sea water. *The Journal of Experimental Biology,* **203**: 213–220.

Florkin, M. & E. Schoffenfiels, 1965. Euryhalinity and the concept of physiological radiation. In: Munday, K.A. (ed.), *Studies on Comparative Biochemistry*. Pergamon Press, London. Pp. 6–40.

Glaessner, M.F., 1969. *Decapoda.* In: Moore, R.C. & R. Part (eds.), *Treatise on Invertebrate Paleontology*. University of Kansas and Geological Society of America, Pp. R400–R651.

Greenaway, P. & R.E. MacMillen, 1978. Salt and water balance in the terrestrial phase of the inland crab *Holthuisana* (*Australothelphusa*) *transversa* Martens (Parathelphusoidea: Sundathelphusidae). *Physiological Zoology,* **51**: 217-229.

Greenaway, P., 1981. Sodium regulation in the freshwater/land crab *Holthuisana transversa*. *Journal of Comparative Physiology B,* **142**: 451–456.

Hall, R., 1998. The plate tectonics of Cenozoic SE Asia and the distribution of land and sea. In: Hall, R. & J.D. Holloway (ed.). *Biogeography and Geological Evolution of SE Asia*. Backhuys Publishers, Leiden, The Netherlands. Pp. 99–131.

Harris, R.R., 1975. Urine production rate and urinary sodium loss in the freshwater crab *Potamon edulis*. *Journal of comparative Biochemistry and Physiology,* **96**: 143–153.

Harris, R.R. & H. Micallef, 1971. Osmotic and ionic regulation in *Potamon edulis*, a fresh- water crab from Malta. *Comparative Biochemistry and Physiology.* **38A**, 769–776.

Holdich, D.M., M.M. Harlioglu, & I. Firkins, 1997. Salinity adaptations of crayfish in British waters with particular reference to *Austropotamobius pallipes, Astacus leptodactylus* and *Pacifastacus leniusculus*. *Estuarine, Coastal and Shelf Science,* **44**: 147–154.

Hopkins, P.M., 1982. Growth and regeneration patterns in the fiddler crab *Uca pugilator*. *Biological Bulletin,* **163**: 301–319.

Im, J.-G., Y. Kong, Y.M. Shin, S.-O. Yang, J.G. Song, M.C. Han & et al., 1993. Pulmonary Paragonimiasis: Clinical and Experimental Studies. *Radiographics,* **13**: 575–586.

Kamemoto, F.I. & J.K. Ono, 1969. Neuroendocrine regulation of salt and water balance in the crayfish *Procambarus clarkii*. *Comparative Biochemistry and Physiology,* **29**: 393–401.

Kendall, A. & F.J. Schwartz, 1964. Salinity tolerances of two Maryland crayfishes. *The Ohio Journal of Science,* **64**: 403–409.

Kirschner, L.B., 1991. Water and ions. In Prosser, C. L. (ed.), *Environmental and metabolic animal physiology*. *Comparative Animal Physiology* (4th ed.) Wiley-Liss, New York. Pp. 13–107.

Kinne, O., 1963. Adaptation, a primary mechanism of evolution. In: Whittington, H.B. & W.D.I. Rolfe (eds.), *Proceedings of a conference held at Cambridge, Massachusetts, March 6 – 8, 1962. Phylogeny and Evolution of Crustacea*. Museum of Comparative Zoology, Special Publications. Pp. 27–50.

Klaus, S., C.D. Schubart & D. Brandis, 2006. Phylogeny, biogeography and a new taxonomy for the Gecarcinucoidea Rathbun, 1904 (Decapoda: Brachyura). *Organism, Diversity & Evolution* **6**: 199-217.

Krishnamoorthy, R.V. & K. Srihari, 1973. Changes in the excretory patterns of the fresh-water field crab *Parathelphusa hydrodromous* upon adaptation to higher salinities. *Marine Biology,* **21**: 341–348.

Lutz, P.L., 1969. Salt and water balance in the west African fresh-water/land crab *Sudanonautes africanus africanus* and the effects of desiccation. *Comparative Biochemistry and Physiology,* **30**: 469-480.

Martin, J.W. & G.E. Davis, 2001. *An updated classification of the recent Crustacea*. Natural History Museum of Los Angeles County. Los Angeles, California.

Martinez, A.-S., J.-Y. Toullec, B. Shillito, M. Charmantier-Daures & G. Charmantier, 2001. Hydromineral Regulation in the Hydrothermal Vent Crab *Bythograea thermydron*. *Biological Bulletin.*, **201**: 167–174.

Menard, S., 2001. *Applied Logistic Regression 2nd edition*. Sage University Papers Series on Quantitive Applications in the Social Sciences 07-106. Sage, Thousand Oaks, California. 111 pp.

Metcalfe, I., 1998. Palaeozoic and Mesozoic geological evolution of the SE Asian region: multidisciplinary constraints and implications for biogeography. In: Hall, R. & Holloway, J.D. (eds.), *Biogeography and Geological Evolution of SE Asia*. Backhuys Publishers, Leiden, The Netherlands. Pp. 25–41.

Mey, W., 1998. *The distribution of Apsilochorema Ulmer, 1907: biogeographic evidence for the Mesozoic accretion of a Gondwana microcontinent to Laurasia.* In: Hall, R. & Holloway, J.D. (eds.), *Biogeography and Geological Evolution of SE Asia*. Backhuys Publishers, Leiden, The Netherlands. Pp. 91–98.

Mills, B.J. & M.C. Geddes, 1980. Salinity tolerance and osmoregulation of the Australian freshwater crayfish *Cherax destructor* Clark (Decapoda: Parastacidae). *Australian Journal of Marine and Freshwater Research,* **31** (5), 667–676.

Morris, S., 2001. Neuroendocrine regulation of osmoregulation and the evolution of air-breathing in decapod crustaceans. *Journal of Experimental Biology,* **204**: 979–989.

Morris, S., 2002. The ecophysiology of air-breathing in crabs with special reference to *Gecarcoidea natalis*. *Comparative Biochemistry and Physiology Part B,* **131**: 559-570.

Morris, S. & W.J. van Aardt, 1998. Salt and water relations, and nitrogen excretion, in the amphibious African freshwater crab *Potamonautes warreni* in water and in air. *The Journal of Experimental Biology,* **201**: 883-893.

Myers, G.S., 1951. Fresh-water fishes and East Indian zoogeography. *Stanford Ichthyology Bulletin*, **4**: 11–21.

Ng, P.K.L. & G. Rodriguez, 1995. Freshwater crabs as poor zoogeographical indicators: a critique of Banarescu (1990). *Crustaceana*, **68**: 636–645.

Ng, P.K.L., Z. Stevcic & G. Pretzmann, 1995. A revision of the familiy Deckenidae Ortmann, 1897 (Crustacea: Decapoda: Brachyura: Potamoidea), with description of a new genus (Gecarcinucoidea: Gecarcinucidae) from the Seychelles, Indian Ocean. *Journal of Natural History (London),* **29**: 581-600.

Onken, H., 1996. Active and electrogenic absorption of Na^+ and Cl^- across posterior gills of *Eriocheir sinensis*: influence of short-term osmotic variations. *Journal of Experimental Biology*, **199**: 901–910.

Onken, H. & J.C. McNamara, 2002. Hyperosmoregulation in the red freshwater crab *Dilocarcinus pagei* (Brachyura, Trichodactylidae): structural and functional asymmetries of the posterior gills. *Journal of Experimental Biology*, **205**: 167–175.

Onken, H., A. Schöbel, J. Kraft, & M. Putzenlechner, 2000. Active NaCl absorption across split lamellae of posterior gills of the Chinese crab *Eriocheir sinensis*: Stimulation by eyestalk extract. *The Journal of Experimental Biology,* **203**: 1373–1381.

Padmanabhanaidu, B. & R. Ramamurthy, 1961. The influence of sex and size on the osmotic pressure, the chloride and the free amino acids of the blood of the freshwater field crab, *Parathelphusa sp.* and the freshwater mussel, *Lamellidens marginalis*. *Journal of Experimental Biology,* **38**: 35–41.

Pequeux, A., 1995. Osmotic regulation in crustaceans. *Journal of Crustucean Biology.,* **15**: 1–60.

Pequeux, A. & R. Gilles, R., 1981. Na^+ fluxes across isolated perfused gills of the Chinese crab *Eriocheir sinensis*. *Journal of Experimental Biology*, **92**: 173–186.

Pequeux, A., R. Gilles, & W.S. Marshall, 1988. NaCl transport in gills and related structures. In: Greger, R. (ed.), *Advances in Comparative and Environmental Physiology,* vol. 1. Springer, Berlin, Heidelberg. Pp. 1–73.

Pretzmann, G., 1973. Grundlagen und Ergebnisse der Systematik der Pseudothelphusidae. *Zeitschrift fuer Zoologische Systematik und Evolutionsforschung*, **11**: 196-218.

Randall, R., W. Burggren, & K. French, 2001. *Ionic and Osmotic Balance.* In: *Eckert Animal Physiology – Mechanisms and Adaptations* (5^{th} ed.). W.H. Freeman and Company, New York. Pp. 579–630.

Riestenpatt, S., W. Zeiske, & H. Onken, 1994. Cyclic AMP stimulation of electrogenic uptake of Na^+ and Cl^- across the gill epithelium of the Chinese crab *Eriocheir sinensis*. *Journal of Experimental Biology,* **188**: 159–174.

Rodriguez, G., 1986. Centers of radiation of freshwater crabs in the Neotropics. *Crustacean Issues,* **4**: 51-67.

Rothman, K.J., 2002. *Measuring Disease Occurrence and Causal Effects*. In: *Epidemiology An Introduction.* Oxford University Press, New York. Pp. 24-56.

Schlieper, C. & F. Herrmann, 1930. Beziehungen zwischen Bau und Funktion bei den Exkretionsorganen dekapoder Crustaceen. *Zoologische Jahrbuecher Abteilung fuer Anatomie und Ontogenie der Tiere*, **52**: 624–630.

Scholles, W., 1933. Ueber die Mineralregulation wasserlebender Evertebraten. *Journal of Comparative Physiology A*, **19**: 183–236.

Schubart, C.D. & R. Diesel, 1998. Osmoregulatory capacities and penetration into terrestrial habitats: a comparative study of Jamaican crabs of the genus *Armases* Abele, 1992 (Brachyura: Grapsidae: Sesarminae). *Bulletin of Marine Science,* **62**: 743–752.

Schubart, C.D. & R. Diesel, 1999. Osmoregulation and the transition from marine to freshwater and terrestrial life: a comparative study of Jamaican crabs of the genus *Sesarma*. *Archiv fuer Hydrobiologie,* **145**: 331–347.

Science, Technology and Law Panel, Policy and Global Affairs, 2002. *The Scientist's Role in the Courtroom*. In: *The Age of Expert Testimony: Science in the Courtroom, Report of a Workshop*. National Research Council, Washington D.C. Pp. 15 -21.

Shaw, J., 1958a. Solute and water balance in the muscle fibres of the East African fresh-water crab, *Potamon niloticus* (M. Edw.) *Journal of Experimental Biology,* **36**: 145-156.

Shaw, J., 1958b. Salt and water balance in the East African fresh-water crab, *Potamon niloticus* (M. Edw.) *Journal of Experimental Biology,* **36**: 157-176.

Shaw, J., 1962. Kinetic aspects of ion regulation in aquatic animals. In: Carthy, J.D. and C.Duddington (eds.), *Viewpoints in Biology* Vol. 2. London: Butterworth. Pp. 163–201.

Shih, H.-T, G.X. Chen, & L.M. Wang, 2005. A new species of freshwater crab (Decapoda: Brachyura: Potamidae) from Dongyin Island, Matsu, Taiwan, defined by morphological and molecular characters, with notes on its biogeography. *Journal of Natural History,* **39**: 2901–2911.

Shih, S.-T., H.C. Hung, C.D. Schubart, A.C. Chen, & H.W. Chang, 2006. Intraspecific genetic diversity of the endemic freshwater crab *Candidiopotamon rathbunae* (Decapoda, Brachyura, Potamidae) reflects five million years of the geological history of Taiwan. *Journal of Biogeography,* **33**: 980–989.

Sparks, J.S., 2004. Molecular phylogeny and biogeography of the Malagasy and South Asian cichlids (Teleostei: Perciformes: Cichlidae). *Molecular Phylogenetics and Evolution,* **30**: 599–614.

Sternberg, R. von & N. Cumberlidge, 2001. Notes on the position of the true freshwater crabs within the brachyrhynchan Eubrachyura (Crustacea: Decapoda: Brachyura). *Hydrobiologia,* **449**: 21-39.

Sternberg, R. von, N. Cumberlidge, & G. Rodriguez, 1999. On the marine sister groups of the freshwater crabs (Crustacea: Decapoda: Brachyura). *Blackwell Wissenschafts-Verlag*, **37**: 19-38.

Susanto, G.N. & G. Charmantier, 2000. Ontogeny of osmoregulation in the crayfish *Astacus leptodactylus. Physiological and Biochemical Zoology,* **73**: 169–176.

Szombathy, K., 1916. Die tertiaeren Formen der Gattung *Potamon (Thelphusa)* und ihre palaearktischen Nachkommen. *Ann. Mus. Nat. Hung., Budapest*, **14**: 281–472.

Vences, M., J. Freyhof, R. Sonnenberg, J. Kosuch, & M. Veith, 2001. Reconciling fossils and molecules: Cenozoic divergence of cichlid fishes and the biogeography of Madagascar. *Journal of Biogeography*, **28**: 1091–1099.

Venkatachari, S.A.T. & N.E. Ambore, 1973. Effect of size on the protein composition in the tissues of the freshwater crab, *Parathelphusa* (*Barythelphusa*) *guerini* Milne Edwards. *Hydrobiologia,* **43**: 157–169.

Weis, J.S.. 1976. Effects of environmental factors on regeneration and molting in fiddler crabs. *Biological Bulletin*, **150**: 152–163.

Wood, C. & R.G. Boutilier, 1985. Osmoregulation, ionic exchange, blood chemistry, and nitrogenous waste excretion in the land crab *Cardisoma carnifex*: a field and laboratory study. *Biological Bulletin*, **169**: 267–290.

APPENDIX A

Table A1 List of all freshwater crab species in Thailand including broad area (S = South, N = North, E = East, W = West, Thailand = unknown)

Gecarcinucidae			
Genus	**Species**	**Authority**	**Thailand**
Phricotelphusa	*deharvengi*	Ng, 1988	Thailand
Phricotelphusa	*limula*	(Hilgendorf, 1882)	S
Phricotelphusa	*ranongi*	Naiyanetr, 1982	S
Phricotelphusa	*sirindhorn*	Naiyanetr, 1989	S
Phricotelphusa	*aedes*	Kemp. 1923	Thailand
Thaksinthelphusa	*yongchindaratae*	Naiyanetr, 1988	S
Parathelphusidae			
Genus	**Species**	**Authority**	**Thailand**
Mekhongthelphusa	*brandti*	(Bott, 1968)	Thailand
Mekhongthelphusa	*neisi*	(Rathbun, 1902)	Thailand
Esanthelphusa	*chiangmai*	(Ng & Naiyanetr, 1993)	N
Esanthelphusa	*denchaii*	Naiyanetr, 1984	N
Esanthelphusa	*dugasti*	(Rathbun, 1902)	NE
Esanthelphusa	*fangensis*	Naiyanetr .1987	Thailand
Esanthelphusa	*nani*	Naiyanetr, 1984	N
Esanthelphusa	*nimoafi*	Yeo, 2004	Thailand
Esanthelphusa	*phetchaburi*	Ng & Naiyanetr, 1993	C
Heterothelphusa	*beauvoisi*	(Rathbun, 1902)	NE
Mekhongthelphusa	*kengsaphu*	Naiyanetr & Ng, 1995	Thailand
Mekhongthelphusa	*tetragona*	(Rathbun, 1902)	NE
Salangathelphusa	*brevimarginata*	(Hilgendorf, 1882)	SW
Salangathelphusa	*brevicarinata*	Hilgendorf, 1882	SW
Sayamia	*germaini*	(Rathbun, 1902)	Thailand
Sayamia	*sexpunctata*	(Lanchester, 1906)	S
Sayamia	*bangkokensis*	(Naiyanetr, 1982)	C
Siamthelphusa	*faxoni*	(Rathbun, 1905)	C
Siamthelphusa	*holthuisi*	Naiyanetr & Ng, 1991	E
Siamthelphusa	*improvisa*	(Lanchester, 1901)	SE
Siamthelphusa	*nan*	Ng & Naiyanetr, 1997	N
Siamthelphusa	*paviei*	(De Man, 1898)	NW
Siamthelphusa	*retimanus*	Ng & Naiyanetr, 1997	C
Siamthelphusa	*transversa*	Ng & Naiyanetr, 1997	Thailand
Siamthelphusa	*variegata*	Ng & Naiyanetr, 1997	C
Siamthelphusa	*acutidens*	Ng & Naiyanetr, 1997	N
Somanniathelphusa	*juliae*	Bott, 1968	E
Somanniathelphusa	*maehongsonensis*	Naiyanetr, 1987	N
Potamidae			
Genus	**Species**	**Authority**	**Thailand**

Demanietta	*huahin*	Yeo, Naiyanetr & Ng, 1998	S
Demanietta	*khirikhan*	Yeo, Naiyanetr & Ng, 1998	Thailand
Demanietta	*lansak*	Yeo, Naiyanetr & Ng, 1998	C
Demanietta	*manii*	(Rathbun, 1904)	C
Demanietta	*nakhongensi*	Yeo, Naiyanetr & Ng, 1998	S
Demanietta	*renongensis*	(Rathbun, 1904)	S
Demanietta	*smalleyi*	(Bott, 1966)	?
Demanietta	*suanphung*	Yeo, Naiyanetr & Ng, 1998	C
Demanietta	*thagatensis*	(Rathbun, 1904)	C ?
Demanietta	*tritrungensis*	(Naiyanetr, 1986)	C
Demanietta	*tenasserimensis*	De Man, 1898	C
Dromothelphusa	*namuan*	(Naiyanetr, 1993)	NE
Dromothelphusa	*nayung*	(Naiyanetr, 1993)	NE
Dromothelphusa	*pealianoides*	(Bott, 1966)	Thailand?
Dromothelphusa	*sangwan*	Naiyanetr, 1997	N
Dromothelphusa	*longipes*	(A. Milne-Edwards, 1869)	Thailand
Esanpotamon	*namsom*	Naiyanetr & Ng, 1997	NE
Flabellamon	*erawanense*	Naiyanetr, 1992	Thailand
Johora	*thaiana*	Leelawathanagoon, Lheknim & Ng, 2005	S
Kanpotamon	*duangkhaei*	Ng & Naiyanetr, 1993	C
Larnaudia	*chaiyaphumi*	(Naiyanetr, 1982)	NE
Lobothelphusa	*dayamun*	Wood-Mason, 1871	Thailand
Lobothelphusa	*feae*	(de Man, 1898)	Thailand
Lobothelphusa	*gibbosa*	Ng & Kosuge, 1997	Thailand
Neolarnaudia	*botti*	Türkay & Naiyanetr, 1987	Thailand?
Neolarnaudia	*beusekomae*	Bott, 1970	NE
Larnaudia	*larnaudii*	A. Milne-Edwards, 1869	C
Phaibulamon	*stilipes*	Ng, 1992	C
Potamiscus	*andersonianus*	(Wood Mason, 1871)	?
Potamiscus	*doichiangdao*	(Naiyanetr & Ng, 1990)	NW
Potamiscus	*elaphrius*	Dai et al., 1990	?
Potamiscus	*hafniensis*	Bott, 1966	?
Potamiscus	*lipkei*	(Ng & Naiyanetr, 1993)	N
Potamiscus	*luangprabangensis*	(Rathbun, 1904)	NE
Potamiscus	*maehongsonensis*	(Naiyanetr, 1992)	C, NE
Potamiscus	*phuluangensis*	(Bott, 1970)	NE
Potamiscus	*prolatus*	Brandis, 2000	Thailand
Potamiscus	*rongjingense*	Dai et al., 1990	?
Potamon	*boonyaratae*	Naiyanetr, 1987	Thailand
Potamon	*chinghungense*	Dai,Song,He,Cao,Xu & Zhong, 1975	?
Potamon	*cochinchinense*	De Man, 1898	N, E
Potamon	*flexum*	Dai, Song, Li & Liang, 1980	?
Potamon	*hispidum*	(Wood-Mason, 1871)	?
Potamon	*hokuoense*	Dai,Song,He,Cao,Xu & Zhong, 1975	?
Potamon	*jarujini*	Ng & Naiyanetr, 1993	N
Potamon	*loasnesis*	Rathbun, 1904	?

Potamon	*maesariang*	Ng & Naiyanetr, 1993	N
Potamon	*montosus*	Dai,Song,He,Cao,Xu & Zhong, 1975	?
Potamon	*namlang*	Ng & Naiyanetr, 1993	Thailand
Potamon	*phuphanense*	Naiyanetr, 1992	NE
Potamon	*somchaii*	Ng & Naiyanetr, 1993	NE
Potamon	*ubon*	Ng & Naiyanetr, 1993	NE
Potamon	*yotdomense*	Naiyanetr, 1984	NE
Potamon	*doisutep*	Naiyanetr & Ng, 1990	N
Potamon	*nan*	Ng & Naiyanetr, 1993	N
Potamon/Ranguna	*smithianum/a*	Kemp, 1923	NE, E
Pudaengon	*inornatum*	(Rathbun, 1904)	NE, E
Pudaengon	*mukdahan*	Ng & Naiyenetr, 1995	NE
Pudaengon	*sakonnakorn*	Ng & Naiyenetr, 1995	NE
Pudaengon	*thatphanom*	Ng & Naiyenetr, 1995	NE
Pudaengon	*wanonniwat*	Ng & Naiyenetr, 1995	NE
Ranguna	*phrae*	Naiyanetr, 1984	C
Stelomon	*pruinosum*	(Alcock, 1909)	C
Stelomon	*tharnlod*	Yeo & Naiyanetr, 2000	W
Stelomon	*kanchanaburiense*	Yeo & Naiyanetr, 2000	W
Stoliczia	*ekavibhathai*	Ng & Naiyanetr, 1986	SE
Stoliczia	*changmanae*	Ng, 1988	S
Stoliczia	*cognata*	(Roux, 1936)	S
Stoliczia	*holthuisi*	Ng & Yang, 1985	S
Stoliczia	*pahangensis*	(Roux, 1936)	S ?
Stoliczia	*panhai*	Ng & Naiyanetr, 1986	SW
Stoliczia	*perlensis*	(Bott, 1966)	S
Stoliczia	*rafflesi*	(Roux, 1936)	S ?
Takpotamon	*maesotense*	(Naiyanetr, 1992)	N
Terrapotamon	*abbotti*	(Rathbun, 1898)	S
Terrapotamon	*palian*	Ng & Naiyanetr, 1998	S
Stoliczkia	*chaseni*	(Roux, 1934)	S ?
Thaiphusa	*sirikit*	(Naiyanetr, 1992)	W
Thaiphusa	*chantaburiense*	(Chuersri, 1973)	E
Thaipotamon	*dansai*	Ng & Naiyanetr, 1993	NE
Thaipotamon	*lomkao*	Ng & Naiyanetr, 1993	N
Thaipotamon	*siamense*	A. Milne-Edwards, 1869	C
Thaipotamon	*smitinandi*	Naiyanetr & Turkay, 1984	E
Thaipotamon	*sphaeridium*	(Kemp, 1923)	Thailand
Thaipotamon	*varoonphornae*	Ng & Naiyanetr, 1993	E
Thaipotamon	*chulabhorn*	Naiyanetr, 1993	NE
Tomaculamon	*stenixys*	Yeo & Ng, 1997	NW
Tomaculamon	*pygmaeus*	Yeo & Ng, 1997	NW

Table A2 Freshwater crabs of Thailand including ecology

Gecarcinucidae				
Species	**Thailand**	**Province**	**Size of male holotype**	**Ecology**
P. deharvengi	Thailand			cave
P. ranongi	S	Ranong Prov.	15 x 13 mm male lectotype	waterfall
P. sirindhorn	S	Ranong Prov.	20 x 16 mm	waterfall
T. yongchindaratae	S	Phangnga Province	17.7 x 13.2 male lectotype	waterfall
Parathelphusidae				
Species	**Thailand**	**Location**	**Size of male holotype**	**Ecology**
C. brandti	Thailand	Dan Sai, Loei	24 x 18 mm	waterfall
C. neisi	Thailand			
E. chiangmai	N	Chiang Mai, Lamphun & Uttaradit Prov.	43.8 x 33.1 mm	ricefield
E. denchaii	N	Phrae Prov.	44 x 35 mm male lectotype	ricefield
E. dugasti	NE	Nakhon Phanom Prov.	31 x 25 mm largest 43 x 35 mm (male)	ricefield
E. fangensis	N	Chiang Mai Province, Lampang Province	32 x 25 mm	ricefield
E. nani	N	Nan Prov.	39 x 32 mm	ricefield
E. phetchaburi	C	Phetchaburi, Lop Buri, & Prachuap Khiri Khan Prov.	33.5 x 26.1 mm	
H. beauvoisi	NE	Nakhon Ratchasima Prov.	17.2 x 14.3 mm	
M. kengsaphu	Thailand	Mun River	33.7 x 26.5 mm	
M. tetragona	NE	Mekhong River	25 x 22 mm (largest female)	
S. brevimarginata	SW	Phuket-Salanga Island		
S. brevicarinata	SW	Phuket		
S. germaini	Thailand	Saraburi & Ratchaburi Prov.		
S. sexpunctata	S	Sai Kau & Cape Patani		
S. bangkokensis	C	Bangkok Metropolitan area	58 x 45 mm male lectotype	
S. faxoni	C	Lop Buri Province		
S. holthuisi	E	Trat Province	36.5 x 30 mm	
S. improvisa	SE	Trang, Chumpon, Surat Thani Province	37 x 21 mm, largest 39 x 30 mm (male)	shallow fast flowing water, waterfalls
S. nan	N	Nan Province	35.8 x 26.8 mm	
S. paviei	NW	Chiang Mai, Lamphun Province		
S. retimanus	C	several provinces	29.7 x 22.1 mm	
S. transversa	N	Phrae area	29.4 x 22.5 mm	
S. variegata	C		39 x 29.9 mm	
S. acutidens	N	Tak area	31.5 x 25.3 mm	
S. juliae	E	Nakhon Nayok & Chanthaburi	51x 39 mm	
S. maehongsonensis	N	Mae Hong Son Province	23 x 19 mm	

Potamidae				
Species	**Thailand**	**Location**	**Size of male holotype**	**Ecology**
D. huahin	S	Phetchaburi Province	40.7 x 29.4 mm	
D. khirikhan	Thailand		47.3 x 33.9 mm	
D. lansak	C	Ratchaburi, Kanchanaburi & Phetchaburi Prov.	44.8 x 32.4mm	
D. manii	C		47.8 x 35 mm	
D. nakhongensi	S	Nakhons Si Thammarat Prov.	43 x 31.7 mm	
D. renongensis	S	Trang, Krabi, Surat Thani, Phuket, Phangnaga, Ranong Chumphon Prov.		
D. smalleyi	island	Ko chang	38 x 28 mm	
D. suanphung	C	Ratchaburi & Kanchanaburi Prov.	44.9 x 32.8 mm	
D. tritrungensis	C	Kanchanaburi Prov.	54.2 x 40.1 mm	waterfall
D. tenasserimensis	C	Bangkok Area		
D. namuan	NE	Nan Prov.	51.5 x 40 mm	
D. nayung	NE	Udon Thani Prov.	44 x 35 mm	
D. pealianoides	Thailand?	possibly Laos		
D. sangwan	N	Chang Rai Province	38 x 30 mm	terrestrial
E. namsom	NE	Udon Thani Prov.	31.3 x 22.7 mm	
E. erawanense	Thailand	Kanchanaburi Prov.	34 x 25.3 mm	waterfall
J. thaiana	S	Narathiwat Prov.	26.1 x 22 mm	
K. duangkhaei	C	Kanchanaburi Prov.	49.8 x 35.5 mm	
L. chaiyaphumi	NE	Chaiyaphum Prov.	41 x 31 mm	
N. beusekomae	NE	Khao Yai Nat'l Park	46 x 36 mm	
N. larnaudii	C	Near Bangkok	45 x 38 mm	
P. stilipes	C	Kanchanaburi Prov.	28.5 x 22.7 mm	cave
P. doichiangdao	NW	Chiang Mai prov. Mountains	50.5 x 38 mm	
P. lipkei	N	Chiang Rai Prov.	56.8 x 42.8 mm	
P. maehongsonensis	C, NE	Mae Hong Son Prov.	39 x 30.2 mm	
P. phuluangensis	NE	Phrae, Phetchabun, Udon Thani, Phu Luang Prov.	53 x 40 mm	
P. boonyaratae	Thailand	Trat Prov.	46 x 38 mm	
P. cochinchinense	N, E	Chanthaburi & Chiangmai Prov.	50 x 43 mm	
P. jarujini	N	Chiang Mai prov.	50 x 38.5 mm	
P. maesariang	N	Mae Hong Son Prov.	47 x 36 mm	waterfall
P. namlang	Thailand	Mae Hong Son Prov.	44.5 x 35 mm	cave
P. phuphanense	NE	Sakon Nakhon Prov.	32.6 x 26.5 mm	
P. somchaii	NE	Sakon Nakhon Prov.	32 x 26.5 mm	
P. ubon	NE	Ratchathani Prov.	49 x 39 mm	
P. yotdomense	NE	Ubon Ratchathani Prov.	47 x 36 mm	
P. doisutep	N	Chiang Mai Prov.		
P. nan	N	Nan Prov.	35.6 x 27.2 mm	
P. smithianum/a	NE, E	Chanthaburi, Khao Sebap/Khao So Tao Tai, Khao Yai Nat'l Park	57 x 45 mm	

P. inornatum	NE, E		48 x 38 mm	
P. sakonnakorn	NE	Amphoe Muang		
P. thatphanom	NE	Amhoe That Phanom	56.1 x 44.5 mm	
R. phrae	C	Phrae Prov.	55 x 2 mm	
S. pruinosum	C	Phetchaburi Prov.		
S. tharnlod	W	Kanchanaburi Prov.	38.5 x 29.1 mm	
S. kanchanaburiense	W	Kanchanaburi Prov.	62.4 x 47.7 mm	waterfall
S. ekavibhathai	SE	Pattani, Narathiwat, & Yala Prov.	30.8 x 23.5 mm	
S. pahangensis	S ?	Malaysia?		1900m
S. panhai	SW	Songkhla & Satun Prov.		waterfall
T. maesotense	N	Tak Province	46.5 x 35.3 mm	
T. abbotti	S	Trang	45 x 35 mm (male lectotype)	
T. palian	S	Trang Province		
T. chaseni	S ?	Pahang ? Malaysia		1300m
T. sirikit	W	Kanchanaburi Prov.	33 x 48 mm	burrows
T. chantaburiense	E	Chanthaburi		
T. dansai	NE	Loei Prov.	48.5 x 34.5 mm	
T. lomkao	N	Phetchaboon Prov.	43.5 x 32 mm	burrows in forest
T. siamense	C	Kan Suri, Chanthaburi, Kanchanaburi, near Bangkok		
T. smitinandi	E	Chanthaburi Prov.	43.3 x 31.6 mm	
T. varoonphornae	E	Prachin Buri Prov.	43.5 x 32.3 mm	
T. chulabhorn	NE	Maha Sarakhai Prov.	40 x 31 mm	
T. beusekomae	Thailand	Khao Yai Nat'l Park		
T. stenixys	NW	Tak, Phitsanulok Prov.		
T. pygmaeus	NW	Phitsanulok Prov.		

Table A3 Banarescu's division of freshwater macrodecapods (Banarescu, 1990)

Primary freshwater	
Superfamily Astacoidea	Northern Crayfishes
Superfamily Parastacoidea	Southern Crayfishes
Superfamily Galathoidea	Incl. porcelain crabs
Secondary freshwater: True FW crabs	**Based on Bott (1969)**
Superfamily Parathelphusoidea - Old World - Gecarcinucidae - Sundathelphusidae - Parathelphusidae Superfamily Potamoidea - Potamonautidae - Deckeniidae - Potamidae - Sinopotamidae - Isolapotamidae Superfamily Pseudothelphusoidea - Pseudothelphusidae - (Potamocarcinidae) Superfamily Trichodactyloidea - Trichodactylidae	All Brachyura
Families predominantly marine that include FW genera/species	**Crabs**
- Paguridae	Anomura
- Portunidae	Brachyura
- Hymenosomatidae	Brachyura
- Xanthidae	Brachyura
- Grapsidae	Brachyura
- Ocypodidae	Brachyura

Repeated Measures

There are several assumptions that need to be met for this multivariate ANOVA. First, Box's M Test tests the null hypothesis that the observed covariance matrices of the dependent variables (in this case the osmolality readings of the different days) are equal across groups, and any differences would mean that the null hypothesis should be rejected (SPSS). Second, Mauchly's Test of Sphericity tests circularity and requires that the correlation between the group data should be the same between all groups. If the sphericity assumption is met, then one looks in the table at the value behind the sphericity assumed, if the sphericity assumption is not met, then one has to look at an adjustment value called lower bound Epsilon (SPSS).Violation of sphericity results in a type I error greater than the specified α and means that one has rejected a null hypothesis, when it

was true. Thirdly, Levene's Test tests the ANOVA assumption that the data in each cell comes from populations with the same variance. It tests the null hypothesis that the error variance of the dependent variable, in this case osmolality, is equal across groups. The F statistic and the numerator (df1) and denominator (df2) degrees of freedom are used to calculate the significance value. In order to not reject the null hypothesis and assume equal variance across groups the significance values should not be < 0.05 (SPSS).

Even if one of the assumptions was not met the repeated measures ANOVA was still used because the Box's M Test is a conservative test that rejects the null hypothesis too often and it is also highly sensitive to violations of multivariate normality (homepage www 2 .chass.ncsu.edu/garson/pa765/assumpt.htm #transforms). Log-transforming the data did not help to meet the assumption of equal cell variances, thus the violation of this assumption in certain cases was acknowledged and the results were reviewed with caution.

SPSS produces numerous large tables for its repeated measures ANOVA output - the Multivariate test table, the test of Within-subjects effects and the test of Between-subjects effects. The Multivariate test table gives the value of the F statistic, which is transformed from the test statistic and indicates an approximate F distribution. For an effect such as the repeated measures analysis, osmolality over time, or an interaction of the repeated measures with another of the main or fixed effects to be significant it needs to have F statistic $p < 0.05$. There are several test statistics available. The Pillai's Trace is the most robust and powerful criterion for some practical situations (SPSS). Roy's Largest Root is used when there were not enough degrees of freedom to calculate Pillai's Trace. The table of the test of Within-subjects effects shows the results of the univariate

test for the Within-subjects factor time and possible interaction terms such as length of time exposed vs any of the factors such as salinity, gender or age. In those cases where sphericity could not be assumed the lower-bound epsilon is used, which is the most conservative approach that takes the reciprocal of the degrees of freedom for the Within-subjects factor. The Between-subjects effects table is basically the ANOVA table and this indicates whether fixed effects are significant ($p < 0.05$) or not.

Logistic regression

Logistic regression uses a maximum likelihood technique and is an iterative process (Menard 2001). Logistic regression starts with none of the variables entered into the model (step 0). During the 1st step the variable with the highest score (if significant) is included by forward stepwise regression methods. By default, a score is considered significant if its p-value is less than 0.05 (SPSS). The parameters of the model are summarized in a table where B is the estimated coefficient, with standard error (S.E.) The ratio of B to S.E. squared equals the Wald statistic. If the Wald statistic is significant (< 0.05) then the parameter is useful to the model. However, the Wald Statistic has a disadvantage because for large B's because the estimated standard error is inflated which results in failure to reject the null hypothesis when the null hypothesis is false (type 2 error) (Menard 2001). Exp (B) is the predicted change in odds for a unit increase in the predictor. When Exp (B) is less than 1, increasing values of the variable correspond to decreasing odds of the event's occurrence. When Exp (B) is greater than 1, increasing values of the variable correspond to increasing odds of the event's occurrence. After the model has been produced the loss attributed to removing that component is computed for

each variable in the model at each step. The more a variable contributes to the model the larger the change in -2 log-likelihood (-2LL) (SPSS).

A classification table is used to describe the performance of the final model by cross tabulating the observed response categories with the predicted response categories. In each case, the predicted response is the category treated as 1, if that category's predicted probability is greater than the user-specified cutoff and a correct percentage of the overall model is given for each step.

Another way to describe the performance of the model is by using the Nagelkerke R^2, a pseudo R square. In linear regression, the R-square statistic measures the proportion of the variation in the response that is explained by the model. As the R-square statistic cannot be exactly computed for logistic regression models, pseudo approximations are computed instead. Larger pseudo R-square statistics indicate that more of the variation is explained by the model, to a maximum of 1. However, large R-square numbers as are usually found in linear regression are very uncommon (SPSS).

APPENDIX B

1st GLM test: Box's M Test assumption of equality of covariance matrices was not met (p = 0.009), but the Mauchly's Test assumption of sphericity was met (df = 2, p = 0.743). Levene's Test assumption of equality of error variances was only met after 13 days (p = 0.076), but not after 5 days (p = 0.006) or 9 days (p = 0.038).

Table B1 Multivariate Test of Repeated Measures ANOVA using osmolality data of days 5, 9 and 13 of *E. dugasti* of both genders subjected to different salinities

Effect		*Value*	*F*	*Hyp. df*	*Error df*	*Sig.*	*Observed Power(a)*
	Pillai's Trace	0.50	13.90	2	28	0.00 *	1.00
day	Roy's Largest Root	0.99	13.90	2	28	0.00 *	1.00
day*gender	Pillai's Trace	0.01	0.11	2	28	0.90	0.06
	Roy's Largest Root	0.01	0.11	2	28	0.90	0.06
day*salinity	Pillai's Trace	0.88	4.53	10	58	0.00 *	1.00
	Roy's Largest Root	1.18	6.84	5	29	0.00 *	0.99
day*gender	Pillai's Trace	0.31	1.32	8	58	0.25	0.55
***salinity**	Roy's Largest Root	0.22	1.56	4	29	0.21	0.42

Multivariate Design: Intercept+gender+salinity+gender * salinity. Within Subjects Design: day

Table B2 Test of Within-Subjects Effects of Repeated Measures ANOVA using osmolality data of days 5, 9 and 13 of *E. dugasti* of both genders subjected to different salinities

Source		*Type I Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*	*Observed Power*
	Sphericity Assumed	54582.02	2	27291.01	16.49	0.00*	1.00
day	Lower-bound	54582.02	1	54582.02	16.49	0.00*	0.98
	Sphericity Assumed	412.63	2	206.32	0.12	0.88	0.07
day*gender	Lower-bound	412.63	1	412.63	0.12	0.73	0.06
	Sphericity Assumed	84271.27	10	8427.13	5.09	0.00 *	1.00
day*salinity	Lower-bound	84271.27	5	16854.25	5.09	0.00 *	0.96
	Sphericity Assumed	17288.31	8	2161.04	1.31	0.26	0.54
day*gender *salinity	Lower-bound	17288.31	4	4322.08	1.31	0.29	0.35
	Sphericity Assumed	95994.44	58	1655.08			
Error(day)	Lower-bound	95994.44	29	3310.15			

Table B3 Tests of Between-Subjects Effects of Repeated Measures ANOVA using osmolality data of days 5, 9 and 13 of *E. dugasti* of both genders subjected to different salinities

Source	Type I Sum of Squares	df	Mean Square	F	Sig.	Observed Power
Intercept	33662435.41	1	33662435.41	15749.81348	0.00 *	1
gender	41587.55	1	41587.55	19.46	0.00 *	0.99
salinity	2728011.40	5	545602.28	255.27	0.00 *	1
gender* salinity	6173.61	4	1543.40	0.72	0.58	0.20
Error	61982.36	29	2137.32			

Table B4 Multiple comparisons of Repeated Measures ANOVA using osmolality data of days 5, 9 and 13 of *E. dugasti* of both genders subjected to different salinities

	(I) salinity	(J) salinity	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	0ppt	7ppt	-15.03	15.26	0.92	-61.56	31.49
		15ppt	-93.73	13.17	0.00 *	-133.89	-53.57
		22ppt	-205.56	13.17	0.00 *	-245.72	-165.40
		28ppt	-326.78	15.26	0.00 *	-373.31	-280.26
		30ppt	-387.71	12.51	0.00 *	-425.86	-349.56
	7ppt	0ppt	15.03	15.26	0.92	-31.49	61.56
		15ppt	-78.69	17.23	0.00 *	-131.22	-26.17
		22ppt	-190.53	17.23	0.00 *	-243.05	-138.00
		28ppt	-311.75	18.87	0.00 *	-369.29	-254.21
		30ppt	-372.68	16.73	0.00 *	-423.68	-321.68
	15ppt	0ppt	93.73	13.17	0.00 *	53.57	133.89
		7ppt	78.69	17.23	0.00 *	26.17	131.22
		22ppt	-111.83	15.41	0.00 *	-158.81	-64.86
		28ppt	-233.06	17.23	0.00 *	-285.58	-180.53
		30ppt	-293.98	14.85	0.00 *	-339.25	-248.71
	22ppt	0ppt	205.56	13.17	0.00 *	165.40	245.72
		7ppt	190.53	17.23	0.00 *	138.00	243.05
		15ppt	111.83	15.41	0.00 *	64.86	158.81
		28ppt	-121.22	17.23	0.00 *	-173.75	-68.70
		30ppt	-182.15	14.85	0.00 *	-227.42	-136.88
	28ppt	0ppt	326.78	15.26	0.00 *	280.26	373.31
		7ppt	311.75	18.87	0.00 *	254.21	369.29
		15ppt	233.06	17.23	0.00 *	180.53	285.58
		22ppt	121.22	17.23	0.00 *	68.70	173.75
		30ppt	-60.93	16.73	0.01 *	-111.93	-9.93
	30ppt	0ppt	387.71	12.51	0.00 *	349.56	425.86
		7ppt	372.68	16.73	0.00 *	321.68	423.68
		15ppt	293.98	14.85	0.00 *	248.71	339.25
		22ppt	182.15	14.85	0.00 *	136.88	227.42
		28ppt	60.93	16.73	0.01 *	9.93	111.93
Dunnett t (2-sided) (a)	7ppt	0ppt	15.03	15.26	0.83	-26.38	56.45
	15ppt	0ppt	93.73	13.17	0.00 *	57.98	129.47
	22ppt	0ppt	205.56	13.17	0.00 *	169.81	241.31

	28ppt	0ppt	326.78	15.26	0.00 *	285.37	368.20
	30ppt	0ppt	387.71	12.51	0.00 *	353.75	421.67

Based on observed means. a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

2nd GLM test: Box's M Test assumptions of equality of covariance matrices was met (p = 0.411). The assumption of sphericity could not be tested because there were not enough degrees of freedom to calculate. Levene's test assumption of equality of error variances was not met on day 5 (p = 0.005), but was met on day 9 (p = 0.219).

Table B5 Multivariate Test of Repeated Measures ANOVA using osmolality data of days 5 and 9 of *E. dugasti* sub-adults of both genders in different water depths

Effect		Value	F	Hyp. df	Error df	Sig.	Observed Power(a)
day	Pillai's Trace	0.06	6.67	1	103	0.01 *	0.73
	Roy's Largest Root	0.06	6.67	1	103	0.01 *	0.73
day*gender	Pillai's Trace	0.01	1.34	1	103	0.25	0.21
	Roy's Largest Root	0.01	1.34	1	103	0.25	0.21
day*salinity	Pillai's Trace	0.27	4.83	8	103	0.00 *	1.00
	Roy's Largest Root	0.38	4.83	8	103	0.00 *	1.00
day* waterdepth	Pillai's Trace	0.00	0.06	1	103	0.81	0.06
	Roy's Largest Root	0.00	0.06	1	103	0.81	0.06
day*gender* salinity	Pillai's Trace	0.03	0.71	5	103	0.62	0.25
	Roy's Largest Root	0.03	0.71	5	103	0.62	0.25
Day*gender* waterdepth	Pillai's Trace	0.02	1.82	1	103	0.18	0.27
	Roy's Largest Root	0.02	1.82	1	103	0.18	0.27
day*salinity *waterdepth	Pillai's Trace	0.01	0.57	1	103	0.45	0.12
	Roy's Largest Root	0.01	0.57	1	103	0.45	0.12

Design: Intercept+gender+salinity+waterdepth+gender * salinity+gender * waterdepth+salinity * * waterdepth. Within Subjects Design: day

Table B6 Test of Within-Subjects Effects of Repeated Measures ANOVA using osmolality data of days 5 and 9 of *E. dugasti* adults of both genders in different water depths

Source		*Type I Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*	*Observed Power*
day	Sphericity Assumed	11307.05	1	11307.05	6.67	0.01 *	0.73
	Lower-bound	11307.05	1	11307.05	6.67	0.01 *	0.73
day*gender	Sphericity Assumed	2268.50	1	2268.50	1.34	0.25	0.21
	Lower-bound	2268.50	1	2268.50	1.34	0.25	0.21
day*salinity	Sphericity Assumed	65441.35	8	8180.17	4.83	0.00 *	1.00
	Lower-bound	65441.35	8	8180.17	4.83	0.00 *	1.00
day* waterdepth	Sphericity Assumed	95.86	1	95.86	0.06	0.81	0.06
	Lower-bound	95.86	1	95.86	0.06	0.81	0.06
day*gender* salinity	Sphericity Assumed	6012.40	5	1202.48	0.71	0.62	0.25
	Lower-bound	6012.40	5	1202.48	0.71	0.62	0.25
day*gender* waterdepth	Sphericity Assumed	3075.24	1	3075.24	1.82	0.18	0.27
	Lower-bound	3075.24	1	3075.24	1.82	0.18	0.27
day*salinity* waterdepth	Sphericity Assumed	961.10	1	961.10	0.57	0.45	0.12
	Lower-bound	961.10	1	961.10	0.57	0.45	0.12
Error(day)	Sphericity Assumed	174503.67	103	1694.21			
	Lower-bound	174503.67	103	1694.21			

Table B7 Test of Between-Subjects Effects of Repeated Measures ANOVA using osmolality data of days 5 and 9 of *E. dugasti* sub-adults of both genders in different water depths

Source	*Type I Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*	*Observed Power*
Intercept	61797590.27	1	61797590.27	31516.38	0.00 *	1
gender	13931.93	1	13931.93	7.11	0.01 *	0.75
salinity	4339888.018	8	542486.00	276.66	0.00 *	1
waterdepth	1209.33	1	1209.33	0.62	0.43	0.12
gender*salinity	11361.66	5	2272.33	1.16	0.33	0.40
gender* waterdepth	1277.69	1	1277.70	0.65	0.42	0.13
salinity* waterdepth	5855.52	1	5855.52	2.99	0.09	0.40
Error	201963.29	103	1960.80			

Table B8 Multiple comparisons (of salinity groups) of Repeated Measures ANOVA using osmolality data of days 5 and 9 of *E. dugasti* sub-adults of both genders in different water depths

	(I) salinity	(J) salinity	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	0ppt	7ppt	17.09	11.04	0.83	-17.91	52.08
		15ppt	-55.65	9.03	0.00	-84.27	-27.03
		22ppt	-180.69	11.53	0.00	-217.22	-144.17
		25ppt	-243.29	16.40	0.00	-295.26	-191.32
		28ppt	-304.60	12.10	0.00	-342.95	-266.25
		30ppt	-340.41	9.45	0.00	-370.35	-310.47
		33ppt	-458.91	16.40	0.00	-510.89	-406.94
		13ppt	-66.56	9.69	0.00	-97.27	-35.84
	7ppt	0ppt	-17.09	11.04	0.83	-52.08	17.91
		15ppt	-72.74	12.48	0.00	-112.28	-33.19
		22ppt	-197.78	14.39	0.00	-243.37	-152.19
		25ppt	-260.38	18.52	0.00	-319.07	-201.68
		28ppt	-321.69	14.85	0.00	-368.75	-274.62
		30ppt	-357.50	12.78	0.00	-398.01	-316.99
		33ppt	-476.00	18.52	0.00	-534.70	-417.30
		13ppt	-83.64	12.96	0.00	-124.72	-42.56
	15ppt	0ppt	55.65	9.03	0.00	27.03	84.27
		7ppt	72.74	12.48	0.00	33.19	112.28
		22ppt	-125.04	12.91	0.00	-165.94	-84.14
		25ppt	-187.64	17.40	0.00	-242.78	-132.50
		28ppt	-248.95	13.42	0.00	-291.49	-206.41
		30ppt	-284.76	11.09	0.00	-319.91	-249.62
		33ppt	-403.26	17.40	0.00	-458.40	-348.13
		13ppt	-10.91	11.30	0.99	-46.72	24.90
	22ppt	0ppt	180.69	11.53	0.00	144.17	217.22
		7ppt	197.78	14.39	0.00	152.19	243.37
		15ppt	125.04	12.91	0.00	84.14	165.94
		25ppt	-62.60	18.82	0.03	-122.22	-2.97
		28ppt	-123.91	15.21	0.00	-172.12	-75.70
		30ppt	-159.72	13.20	0.00	-201.56	-117.89
		33ppt	-278.22	18.82	0.00	-337.85	-218.60
		13ppt	114.13	13.38	0.00	71.74	156.53
	25ppt	0ppt	243.29	16.40	0.00	191.32	295.26
		7ppt	260.38	18.52	0.00	201.68	319.07
		15ppt	187.64	17.40	0.00	132.50	242.78
		22ppt	62.60	18.82	0.03	2.97	122.22
		28ppt	-61.31	19.17	0.05	-122.07	-0.55
		30ppt	-97.12	17.62	0.00	-152.96	-41.29
		33ppt	-215.63	22.14	0.00	-285.78	-145.47
		13ppt	176.73	17.75	0.00	120.48	232.98
	28ppt	0ppt	304.60	12.10	0.00	266.25	342.95
		7ppt	321.69	14.85	0.00	274.62	368.75
		15ppt	248.95	13.42	0.00	206.41	291.49
		22ppt	123.91	15.21	0.00	75.70	172.12

		25ppt	61.31	19.17	0.05	0.55	122.07
		30ppt	-35.81	13.71	0.19	-79.25	7.63
		33ppt	-154.31	19.17	0.00	-215.07	-93.55
		13ppt	238.04	13.88	0.00	194.07	282.02
	30ppt	0ppt	340.41	9.45	0.00	310.47	370.35
		7ppt	357.50	12.78	0.00	316.99	398.01
		15ppt	284.76	11.09	0.00	249.62	319.91
		22ppt	159.72	13.20	0.00	117.89	201.56
		25ppt	97.12	17.62	0.00	41.29	152.96
		28ppt	35.81	13.71	0.19	-7.63	79.25
		33ppt	-118.50	17.62	0.00	-174.33	-62.67
		13ppt	273.86	11.64	0.00	236.99	310.73
	33ppt	0ppt	458.91	16.40	0.00	406.94	510.89
		7ppt	476.00	18.52	0.00	417.30	534.70
		15ppt	403.26	17.40	0.00	348.13	458.40
		22ppt	278.22	18.82	0.00	218.60	337.85
		25ppt	215.63	22.14	0.00	145.47	285.78
		28ppt	154.31	19.17	0.00	93.55	215.07
		30ppt	118.50	17.62	0.00	62.67	174.33
		13ppt	392.36	17.75	0.00	336.11	448.61
	13ppt	0ppt	66.56	9.69	0.00	35.84	97.27
		7ppt	83.64	12.96	0.00	42.56	124.72
		15ppt	10.91	11.30	0.99	-24.90	46.72
		22ppt	-114.13	13.38	0.00	-156.53	-71.74
		25ppt	-176.73	17.75	0.00	-232.98	-120.48
		28ppt	-238.04	13.88	0.00	-282.02	-194.07
		30ppt	-273.86	11.64	0.00	-310.73	-236.99
		13ppt	-392.36	17.75	0.00	-448.61	-336.11
Dunnett t (2-sided) (a)	7ppt	0ppt	-17.09	11.04	0.62	-47.68	13.51
	15ppt	0ppt	55.65	9.03	0.00	30.63	80.67
	22ppt	0ppt	180.69	11.53	0.00	148.76	212.62
	25ppt	0ppt	243.29	16.40	0.00	197.85	288.73
	28ppt	0ppt	304.60	12.10	0.00	271.08	338.13
	30ppt	0ppt	340.41	9.45	0.00	314.24	366.59
	33ppt	0ppt	458.91	16.40	0.00	413.48	504.35
	13ppt	0ppt	66.56	9.69	0.00	39.71	93.41

Based on observed means. a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Univariate Test 1st: Levene's Test assumption of equality of error variances was rejected (p = 0.016), and the variance across cells was not equal.

Table B9 Tests of Between-Subjects Effects of Univariate ANOVA using osmolality data from day 13 of *E. dugasti* of different ages and gender in shallow water

Source	*Type I Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*	*Observed Power*
Corrected Model	2976607.24	14	212614.80	100.82	0.00 *	1.00
Intercept	32953103.69	1	32953103.69	15626.01	0.00 *	1.00
gender	1099.25	1	1099.25	0.52	0.47	0.11
age	18496.26	1	18496.26	8.77	0.00 *	0.83
salinity	2941857.56	5	588371.51	279.00	0.00 *	1.00
gender*age	141.56	1	141.56	0.07	0.80	0.06
age*salinity	7509.59	2	3754.79	1.78	0.17	0.36
gender*salinity	7503.02	4	1875.75	0.89	0.47	0.27
Error	183471.08	87	2108.86			
Total	36113182.00	102				
Corrected Total	3160078.31	101				

R Squared = .942 (Adjusted R Squared = .933)

Table B10 Multiple comparisons (post hoc tests) of Univariate ANOVA using osmolality data from day 13 of *E. dugasti* of different ages and gender in shallow water

	(I) salinity	***(J) salinity***	***Mean Difference (I-J)***	***Std. Error***	***Sig.***	***95% Confidence Interval***	
						Lower Bound	Upper Bound
Tukey HSD	0ppt	7ppt	-88.63	24.27	0.01 *	-159.37	-17.89
		15ppt	-172.55	20.33	0.00 *	-231.81	-113.29
		22ppt	-271.28	11.72	0.00 *	-305.43	-237.12
		25ppt	-338.63	24.27	0.00 *	-409.37	-267.89
		30ppt	-425.27	11.96	0.00 *	-460.13	-390.40
	7ppt	0ppt	88.63	24.27	0.01 *	17.89	159.37
		15ppt	-83.92	29.64	0.06	-170.30	2.47
		22ppt	-182.64	24.55	0.00 *	-254.18	-111.11
		28ppt	-250.00	32.47	0.00 *	-344.63	-155.37
		30ppt	-336.63	24.66	0.00 *	-408.51	-264.76
	15ppt	0ppt	172.55	20.33	0.00 *	113.29	231.81
		7ppt	83.92	29.64	0.06	-2.47	170.30
		22ppt	-98.73	20.66	0.00 *	-158.93	-38.52
		25ppt	-166.08	29.64	0.00 *	-252.47	-79.70
		30ppt	-252.72	20.80	0.00 *	-313.33	-192.11
	22ppt	0ppt	271.28	11.72	0.00 *	237.12	305.43
		7ppt	182.64	24.55	0.00 *	111.11	254.18
		15ppt	98.73	20.66	0.00 *	38.52	158.93
		28ppt	-67.36	24.55	0.08	-138.89	4.18
		30ppt	-153.99	12.51	0.00 *	-190.44	-117.54
	28ppt	0ppt	338.63	24.27	0.00 *	267.89	409.37
		7ppt	250.00	32.47	0.00 *	155.37	344.63
		15ppt	166.08	29.64	0.00 *	79.70	252.47
		22ppt	67.36	24.55	0.08	-4.18	138.89
		30ppt	-86.63	24.66	0.01 *	-158.51	-14.76
	30ppt	0ppt	425.27	11.96	0.00 *	390.40	460.13
		7ppt	336.63	24.66	0.00 *	264.76	408.51
		15ppt	252.72	20.80	0.00 *	192.11	313.33
		22ppt	153.99	12.51	0.00 *	117.54	190.44
		28ppt	86.63	24.66	0.01 *	14.76	158.51
Dunnett t (2-sided) (a)	0ppt	30ppt	-425.27	11.96	0.00 *	-456.40	-394.13
	7ppt	30ppt	-336.63	24.66	0.00 *	-400.82	-272.45
	15ppt	30ppt	-252.72	20.80	0.00 *	-306.84	-198.60
	22ppt	30ppt	-153.99	12.51	0.00 *	-186.54	-121.45
	28ppt	30ppt	-86.63	24.66	0.00 *	-150.82	-22.45

Based on observed means.*The mean difference is significant at the 0.05 level. a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Independent T-test to check water depth, then age and gender differences in adult *E. dugasti*

Table B11 Summary of independent samples t-tests that showed significant differences in hemolymph osmolality between juvenile and sub-adult *E. dugasti*. Equal variances assumed.

	t-test for Equality of Means								
			t (a)	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% CI of the Difference	
								Lower	Upper
FA vs FJ	30 ppt shallow	Day 13	2.39	10	0.04 **	53.67	22.46	3.63	103.70
MA vs MJ	30 ppt deep	Day 9	3.11	15	0.01 **	95.27	30.66	29.91	160.62

Table B12 Variables not in the equation of the logistic regression of survival data of *E. dugasti* after 5 days

Day 5			Score	df	Sig.
Step 0	Variables	salinity	3.928	1	.047 **
		gender1 female	3.826	1	.050
		waterdepth1 shallow	5.082	1	.024 **
		age1	2.398	1	.121
		age*salinity	.266	1	.606
		gender*salinity	.352	1	.553
		waterdepth*salinity	.207	1	.649
		Overall statistic	14.152	7	.049 **

Table B13 Variables not in the equation of the logistic regression of survival data of *E. dugasti* after 9 days

Day 9			Score	df	Sig
Step 0	Variables	Salinity	13.702	1	.000 **
		gender1 female	3.436	1	.064
		waterdepth1 shallow	4.076	1	.043 **
		age1 – adult	30.275	1	.000 **
		age*salinity	.015	1	.901
		gender*salinity	4.419	1	.036 **
		waterdepth*salinity	5.323	1	.021 **
		waterdepth*gender	4.637	1	.031 **
		overall statistics	61.187	8	.000 **

Table B14 Variables not included in the equation of the logistic regression of survival data of *E. dugasti* after 13 days

Day 13			Score	df	Sig.
Step 0	Variables	Salinity	22.066	1	.000 *
		gender1 female	1.796	1	.180
		waterdepth1	2.598	1	.107
		age1 adult	16.366	1	.000 *
		age*salinity	1.710	1	.191
		gender*salinity	10.780	1	.001 *
		waterdepth*salinity	15.254	1	.000 *
		waterdepth*gender	2.771	1	.096
		overall statistic	63.308	8	.000 *

Table B15 Relative risk of dying in *E. dugasti* in increased salinities compared to the control (fresh water) after 5, 9 and 14 days. The relative risk criterion is meaningful > 2 and < 0.5

Water depth	**Salinity in ppt**	**Gender /age**	**DAY 5**	**DAY 9**	**DAY 14**
shallow	7	FA	13.33*	1.44	1.57
shallow	7	MA	0.36*	2.20	2.17*
shallow	13	FA	23.08*	1.11	
shallow	13	MA	0.50	0.90	
shallow	15	FA	14.29*	0.86	0.95
shallow	15	MA	0.67	1.40	2.17*
shallow	22	FA	14.29*	1.71	1.05
shallow	22	MA	1.92	1.85	2.12*
shallow	25	FA	28.57*	1.71	1.24
shallow	28	FA	8.33*	0.80	0.78
shallow	30	FA	1	1.85	1.03
shallow	30	MA	0.36	2.14*	1.79
shallow	33	FA	57.41*	1.89	
deep	30	FA	9.1*	1.85	1.03
deep	30	MA	2.6*	2.14	1.79
shallow	22	FJ	0.13*	0.13*	0.59
shallow	22	MJ	13.33*	13.33*	0.50
shallow	30	FJ	3*	3*	1.50
shallow	30	MJ	13.33*	13.33*	0.25*
shallow	33	MJ	20*	60*	
deep	22	FJ	0.93	3.71*	
deep	22	MJ	10*	25*	
deep	30	MJ	5.88*	29.11*	

Table B16 Relative risk of *E. dugasti* dying in fresh water, 22 or 30 ppt. Comparing risk being in deep water compared to being in shallow water or risk being a juvenile compared to being an adult after 5, 9 and 14 days. The relative risk criterion is meaningful > 2 and < 0.5.

salinity	groups	age/ gender	**Day 5**	**Day 9**	**Day 14**
0	deep vs shallow	FA	1	1	1
0	deep vs shallow	MA	0.39*	1	1
0	shallow	FJ vs FA	7.69*	0.18*	0.41*
0	shallow	MJ vs MA	0.05*	0.03*	1.35
22	shallow	FJ vs FA	0.07*	0.01*	0.23*
22	shallow	MJ vs MA	0.35*	0.22*	0.32*
30	deep vs shallow	FA	9.1*	1.00	1
30	deep vs shallow	MA	2.8*	1.00	1
30	shallow	FJ vs FA	23.08*	0.3*	0.60
30	shallow	MJ vs MA	1.87	0.19*	0.19*
30	deep	MJ vs MA	0.44*	2.21*	

APPENDIX C

1st GLM Repeated Measures ANOVA: Two of the three assumptions of the Repeated Measures ANOVA were met, the assumption that the observed covariance matrices of the dependent variables are equal across groups (the null hypothesis of the Box M Test) was not rejected (p = 0.067). The assumption that the data in each cell come from populations with the same variance was confirmed by Levene's Test (Table B1). The assumption of sphericity could not be tested because due to the lack of degrees of freedom.

Table C1 Levene's Test of Equality of Error Variances of Repeated Measures ANOVA using hemolymph osmolality data of days 5 and 9 of both male and female adult *S. germaini* subjected to different salinities

	F	*df1*	*df2*	*Sig.*
mmol/kg	1.446	6	31	0.229
mmol/kg	2.364	6	31	0.053

Design: Intercept+gender+salinity+waterdepth+gender * salinity+salinity * waterdepth+gender * waterdepth. Within Subjects Design: day

Table C2 Multivariate Tests of Repeated Measures ANOVA using hemolymph osmolality data of days 5 and 9 of both male and female adult *S. germaini* subjected to different salinities

Effect		*Value*	*F*	*Hyp. df*	*Error df*	*Sig.*	*Observed Power*
Day	Pillai's Trace	0.211	8.271	1	31	0.007 *	0.796
	Roy's Largest Root	0.267	8.271	1	31	0.007 *	0.796
Day*gender	Pillai's Trace	0.009	0.291	1	31	0.593	0.082
	Roy's Largest Root	0.009	0.291	1	31	0.593	0.082
Day*salinity	Pillai's Trace	0.334	5.181	3	31	0.005 *	0.889
	Roy's Largest Root	0.501	5.181	3	31	0.005 *	0.889
Day*gender* salinity	Pillai's Trace	0.025	0.392	2	31	0.679	0.107
	Roy's Largest Root	0.025	0.392	2	31	0.679	0.107

Multivariate design: Intercept+gender+salinity+gender * salinity. Within Subjects Design: day

Table C3 Test of Within-Subjects effects of Repeated Measures ANOVA using hemolymph osmolality data of days 5 and 9 of both male and female adult *S. germaini* subjected to different salinities

Source		*Type I Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*	*Observed Power(a)*
day	Sphericity Assumed	10968.01	1	10968.01	8.27	0.01 *	0.796
	Lower-bound	10968.01	1	10968.01	8.27	0.01 *	0.796
day*gender	Sphericity Assumed	386.13	1	386.13	0.29	0.59	0.082
	Lower-bound	386.13	1	386.13	0.29	0.59	0.082
day*salinity	Sphericity Assumed	20609.94	3	6869.98	5.18	0.01 *	0.889
	Lower-bound	20609.94	3	6869.98	5.18	0.01 *	0.889
day*gender* salinity	Sphericity Assumed	1038.71	2	519.35	0.39	0.68	0.107
	Lower-bound	1038.71	2	519.35	0.39	0.68	0.107
Error(day)	Sphericity Assumed	41109.71	31	1326.12			
	Lower-bound	41109.71	31	1326.12			

Table C4 Test of Between-Subjects Effects of Repeated Measures ANOVA using hemolymph osmolality data of days 5 and 9 of both male and female adult *S. germaini* subjected to different salinities

Source	*Type I Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*	*Observed Power(a)*
Intercept	26143824.01	1	26143824.01	13279.10	0.000 *	1
gender	68263.86	1	68263.86	34.67	0.000 *	1
salinity	1259453.44	3	419817.81	213.24	0.000 *	1
gender*salinity	5760.56	2	2880.28	1.46	0.25	0.29
Error	61032.63	31	1968.79			

Table C5 Post hoc tests for salinity, Multiple comparisons of Repeated Measures ANOVA using hemolymph osmolality data of days 5 and 9 of both male and female adult *S. germaini* subjected to different salinities

	(I) salinity	***(J) salinity***	***(I-J) Mean Difference***	***Std. Error***	***Sig.***	***95% Confidence Interval***	
						Lower Bound	Upper Bound
	1 0ppt	3 15ppt	-111.02	15.25	0.00 *	-152.40	-69.64
		4 22ppt	-175.19	15.81	0.00 *	-218.10	-132.28
		7 30ppt	-334.40	13.40	0.00 *	-370.79	-298.02
	3 15ppt	1 0ppt	111.02	15.25	0.00 *	69.64	152.40
		4 22ppt	-64.17	16.24	0.00 *	-108.24	-20.10
		7 30ppt	-223.38	13.91	0.00 *	-261.12	-185.64
	4 22ppt	1 0ppt	175.19	15.81	0.00 *	132.28	218.10
		3 15ppt	64.17	16.24	0.00 *	20.10	108.24
		7 30ppt	-159.21	14.52	0.00 *	-198.63	-119.80
	7 30ppt	1 0ppt	334.40	13.40	0.00 *	298.02	370.79
Tukey		3 15ppt	223.38	13.91	0.00 *	185.64	261.12
HSD		4 22ppt	159.21	14.52	0.00 *	119.80	198.63
Dunnett	1 0ppt	7 30ppt	-334.40	13.40	0.00 *	-367.83	-300.98
t	3 15ppt	7 30ppt	-223.38	13.91	0.00 *	-258.06	-188.71
(2-sided)							
(a)	4 22ppt	7 30ppt	-159.21	14.52	0.00 *	-195.43	-123.00

Based on observed means. a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table C6 Variables in the full logistic regression model of the of survival data of *S. germaini* after 5 days

			Score	df	Sig.
Step 0	Variables	salinity	0.481	1	0.488
		gender1 female	2.056	1	0.152
		Waterdepth1 shallow	4.554	1	0.033
		Gender*salinity	0.003	1	0.957
		Waterdepth*salinity	2.257	1	0.133
		Waterdepth*gender	0.741	1	0.389
	Overall Statistics		11.591	6	0.072

Table C7 Variables in the full logistic regression model of survival data of *S. germaini* after 9 days

			Score	df	Sig.
Step 0	Variables	salinity	1.353	1	.245
		gender1	1.324	1	.250
		waterdepth1 shallow	.278	1	.598
		gender*salinity	2.441	1	.118
		waterdepth*salinity	.682	1	.409
		waterdepth*gender	1.680	1	.195
	Overall Statistics		5.711	6	.456

Table C8 Relative risk of dying in *S. germaini* in increased salinities compared to the control (fresh water) after 5 and 9 days. The relative risk criterion is meaningful > 2 and < 0.5.

Water depth	Salinity in ppt	Gender / age	**DAY 5**	**DAY 9**
shallow	15	MA	**20***	**30***
deep	22	FA	**1**	**1**
deep	22	MA	**1**	**40***
deep	30	FA	**1**	**20***
deep	30	MA	**1**	**30***

APPENDIX D

Repeated measures day 5, 9, 13: Box's M Test assumption of equality of covariance matrices was met (p = 0.248). The Mauchly's Test assumption of sphericity was met (df 2, p = 0.532). The assumption of equality of error variances tested by the Levene's Test was met on all days (day 5 p = 0.845, day 9 p = 0.833, day 13 p = 0.405).

Table D1 Between-Subjects Factors of the repeated measures ANOVA of hemolymph osmolality data of days 5, 9 and 13 of juvenile and adult *P. smithianum* in shallow water of different salinities

	Code	N
water depth	1shallow	38
salinity	1 0 ppt	12
	4 22ppt	10
	6 28 ppt	16
gender	1 F	22
	2 M	16
age	0	3
	1	35

Table D2 Multivariate test of the repeated measures ANOVA of hemolymph osmolality data of days 5, 9 and 13 of juvenile and adult *P. smithianum* in shallow water of different salinities. Design Intercept+depth+salinity+gender+age+depth * salinity+depth * gender+salinity * gender+salinity * age. Within Subjects Design day.

Effect		Value	F	Hyp. df	Error df	Sig.	Observed Power
	Pillai's Trace	0.44	12.00	2	30	0.00 *	0.99
day	Roy's Largest Root	0.80	12.00	2	30	0.00 *	0.99
	Pillai's Trace	0		0	0	.	.
Day*depth	Roy's Largest Root	0	0	2	29	1	0.05
	Pillai's Trace	0.29	2.64	4	62	0.04 *	0.71
Day*salinity	Roy's Largest Root	0.41	6.34	2	31	0.00 *	0.87
	Pillai's Trace	0.07	1.20	2	30	0.32	0.24
Day*gender	Roy's Largest Root	0.08	1.20	2	30	0.32	0.24
	Pillai's Trace	0.03	0.46	2	30	0.63	0.12
Day*age	Roy's Largest Root	0.03	0.46	2	30	0.63	0.12
	Pillai's Trace	0	.	0	0	.	.
Day*depth*salinity	Roy's Largest Root	0	0	2	29	1	0.05
	Pillai's Trace	0	.	0	0	.	.
Day*depth*gender	Roy's Largest Root	0	0	2	29	1	0.05

	Pillai's Trace	0.13	1.09	4	62	0.37		0.32
day*salinity*gender	Roy's Largest Root	0.09	1.38	2	31	0.27		0.27
	Pillai's Trace	0	.(b)	0	0	.	.	
Day*salinity*age	Roy's Largest Root	0	0	2	29	1		0.05

Table D3 Test of Within-Test Subjects Effects of the repeated measures ANOVA of hemolymph osmolality data of days 5, 9 and 13 of juvenile and adult *P. smithianum* in shallow water of different salinities

Source		Type I Sum of Squares	df	Mean Square	F	Sig.	Observed Power
	Sphericity Assumed	99650.75	2	49825.37	14.55	0.000 *	1.00
day	Lower-bound	99650.75	1	99650.75	14.55	0.001 *	0.96
	Sphericity Assumed	0	0	.	.	.	.
day*depth	Lower-bound	0	0	.	.	.	.
	Sphericity Assumed	45889.64	4	11472.41	3.35	0.02 *	0.82
day*salinity	Lower-bound	45889.64	2	22944.82	3.35	0.05 *	0.59
	Sphericity Assumed	7069.93	2	3534.97	1.03	0.36	0.22
day*gender	Lower-bound	7069.93	1	7069.93	1.03	0.32	0.17
	Sphericity Assumed	2847.68	2	1423.84	0.42	0.66	0.11
day*age	Lower-bound	2847.68	1	2847.68	0.42	0.52	0.10
day*depth	Sphericity Assumed	0	0	.	.	.	.
*salinity	Lower-bound	0	0	.	.	.	.
day*depth	Sphericity Assumed	0	0	.	.	.	.
*gender	Lower-bound	0	0	.	.	.	.
day*salinity	Sphericity Assumed	15404.04	4	3851.01	1.12	0.35	0.33
*gender	Lower-bound	15404.04	2	7702.02	1.12	0.34	0.23
day*salinity	Sphericity Assumed	0	0	.	.	.	.
*age	Lower-bound	0	0	.	.	.	.
Error(day)	Sphericity Assumed	212378.61	62	3425.46			
	Lower-bound	212378.61	31	6850.92			

Table D4 Test of between subject effects of the repeated measures ANOVA of hemolymph osmolality data of days 5, 9 and 13 of juvenile and adult *P. smithianum* in shallow water of different salinities

Source	Type I Sum of Squares	df	Mean Square	F	Sig.	Observed Power
Intercept	38837180.01	1	38837180.01	7107.69	0.00 *	1
depth	0.00	0	.	.	.	.
salinity	1143509.80	2	571754.90	104.64	0.00 *	1
gender	3183.22	1	3183.22	0.58	0.45	0.11
age	127.40	1	127.40	0.02	0.88	0.05
depth*salinity	0.00	0	.	.	.	.
depth*gender	0.00	0	.	.	.	.
salinity*gender	15606.48	2	7803.24	1.43	0.26	0.28
salinity*age	0.00	0	.	.	.	.
Error	169387.42	31	5464.11			

Table D5 Multiple comparisons post-hoc tests of the repeated measures ANOVA of hemolymph osmolality data of days 5, 9 and 13 of juvenile and adult *P. smithianum* in shallow water of different salinities

	(I) salinity	(J) salinity	(I-J) Mean Difference	Std. Error	Sig.	95% Confidence Interval	
						Lower	Upper Bound
Tukey HSD	1	4	-139.26	18.27	0.00 *	-184.23	-94.28
		6	-235.72	16.30	0.00 *	-275.83	-195.61
	4	1	139.26	18.27	0.00 *	94.28	184.23
		6	-96.47	17.20	0.00 *	-138.81	-54.12
	6	1	235.72	16.30	0.00 *	195.61	275.83
		4	96.47	17.20	0.00 *	54.12	138.81
Dunnett t (2-sided)(a)	4	1	139.26	18.27	0.00 *	96.94	181.57
	6	1	235.72	16.30	0.00 *	197.98	273.46

Based on observed means. a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Univariate ANOVA of day 5: Levene's Test assumption of equal variance was met (p = 0.465).

Table D6 Between Subjects Effects of the Univariate ANOVA of hemolymph osmolality of day 5 of juvenile and adult *P. smithianum* in shallow and deep water of different salinities

		N
depth	1 shallow	70
	2 deep	39
salinity	1 0 ppt	35
	4 22 ppt	33
	5 25 ppt	14
	6 28 ppt	24
	7 30 ppt	3
gender	1	58
	2	51
age	0	27
	1	82

Table D7 Test of Between Subjects Effects of the Univariate ANOVA of hemolymph osmolality of day 5 of juvenile and adult *P. smithianum* in shallow and deep water of different salinities

Source	Type I Sum of Squares	df	Mean Square	F	Sig.	Observed Power
Corrected Model	915957.98	14	65425.57	12.96	0.00 *	1.00
Intercept	32629098.80	1	32629098.80	6460.93	0.00 *	1.00
depth	886.30	1	886.30	0.18	0.68	0.07
salinity	823383.62	4	205845.91	40.76	0.00 *	1.00
gender	254.10	1	254.10	0.05	0.82	0.06
age	17674.26	1	17674.26	3.50	0.06	0.46
salinity* gender	33290.70	3	11096.90	2.20	0.09	0.54
salinity*age	287.22	1	287.22	0.06	0.81	0.06
depth*age	87.07	1	87.07	0.02	0.90	0.05
depth* salinity	40094.70	2	20047.35	3.97	0.02 *	0.70
Error	474720.23	94	5050.22			
Total	34019777.00	109				
Corrected Total	1390678.20	108				

R Squared = .659 (Adjusted R Squared = .608)

Table D8 Multiple comparisons of the Univariate ANOVA of hemolymph osmolality of day 5 of juvenile and adult *P. smithianum* in shallow and deep water of different salinities

	(I) salinity	(J) salinity	(I-J) Mean Difference	Std. Error	Sig.	95% Confidence Interval	
						Lower	Upper Bound
	1	4	-115.65	17.24	0.00 *	-163.61	-67.68
		5	-138.56	22.47	0.00 *	-201.06	-76.05
		6	-205.80	18.83	0.00 *	-258.19	-153.42
		7	-339.68	42.75	0.00 *	-458.59	-220.77
	4	1	115.65	17.24	0.00 *	67.68	163.61
		5	-22.91	22.67	0.85	-85.96	40.13
		6	-90.16	19.06	0.00 *	-143.18	-37.13
		7	-224.03	42.85	0.00 *	-343.23	-104.84
	5	1	138.56	22.47	0.00 *	76.05	201.06
		4	22.91	22.67	0.85	-40.13	85.96
		6	-67.24	23.90	0.05	-133.72	-0.77
		7	-201.12	45.21	0.00 *	-326.87	-75.36
	6	1	205.80	18.83	0.00 *	153.42	258.19
		4	90.16	19.06	0.00 *	37.13	143.18
		5	67.24	23.90	0.05	0.77	133.72
		7	-133.88	43.52	0.02 *	-254.92	-12.83
	7	1	339.68	42.75	0.00 *	220.77	458.59
		4	224.03	42.85	0.00 *	104.84	343.23
Tukey		5	201.12	45.21	0.00 *	75.36	326.87
HSD		6	133.88	43.52	0.02	12.83	254.92
Dunnett t (2-sided) (a)	4	1	115.65	17.24	0.00 *	72.21	159.08
	5	1	138.56	22.47	0.00 *	81.95	195.16
	6	1	205.80	18.83	0.00 *	158.36	253.24
	7	1	339.68	42.75	0.00 *	231.99	447.36

Based on observed means.* The mean difference is significant at the .05 level. a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table D9 Variables not in the equation of the logistic regression of survival data of *P. smithianum* after 5 days

			Score	df	Sig.
Step 0	Variables	salinity	4.515	1	.034 **
		waterdepth shallow	.820	1	.365
		age	.499	2	.779
		age1 juvenile	.271	1	.603
		age2 adult	.498	1	.480
		overall statistics	7.907	4	.095

Table D10 Variables not in the equation of the logistic regression of survival data of *P. smithianum* after 9 days

			Score	df	Sig.
Step 0	Variables	salinity	.482	1	.487
		waterdepth1 shallow	8.329	1	.004
		age	12.304	2	.002
		age1 juvenile	10.992	1	.001
		age2 adult	9.977	1	.002
		waterdepth*salinity	4.689	1	.030

Table D11 Variables not in the equation of the logistic regression of survival data of *P. smithianum* after 13 days

			score	**df**	**Sig**
Step 0	**variables**	salinity	2.224	1	.136
		age	22.494	2	*.000
		age1 juvenile	21.677	1	*.000
		age2 adult	13.312	1	*.000
		waterdepth*salinity	2.224	1	.136

Table D12 Relative risk of dying in *P. smithianum* in increased salinities compared to the control (fresh water) after 5, 9 and 14 days. The relative risk criterion is meaningful > 2 and < 0.5.

Water depth	**Salinity in ppt**	**Gender / age**	**DAY 5**	**DAY 9**	**DAY 14**
shallow	22	FA	0.05*	1.67	1.04
shallow	22	MA	0.03*	0.67	1
shallow	25	FA			
shallow	28	FA	0.05*	0.39*	0.39*
shallow	28	MA	0.03*	0.37*	0.56
deep	25	FA	0.05*	0.56	
deep	25	MA	0.03*	0.03*	
deep	30	FA	2	2	
deep	30	MA	1.67	1.67	
shallow	22	FJ	0.36*	1.19	
shallow	22	MJ	0.42*	0.83	

APPENDIX E

1st GLM Repeated Measures ANOVA: Box's M Test assumption of equality of covariance matrices is met (p = 0.239). The assumption of sphericity cannot be verified as there were not enough degrees of freedom. Levene's test assumption of equality of error variances was met after 5 days (df = 5, p = 0.408) and 9 days (df = 5, p = 0.086).

Table E1 Multivariate Tests of Repeated Measures ANOVA of hemolymph osmolality data of days 5 and 9 of *L. chaiyaphumi* in 22ppt in shallow water and a covariate in 0 ppt

Effect		*Value*	*F*	*Hyp. df*	*Error df*	*Sig.*	*Observed Power*
Day	Pillai's Trace	0.87	27.80	1	4	0.01 *	0.97
	Roy's Largest Root	6.95	27.80	1	4	0.01 *	0.97
Day* covariate	Pillai's Trace	0.64	6.96	1	4	0.06	0.52
	Roy's Largest Root	1.74	6.96	1	4	0.06	0.52
Day*gender	Pillai's Trace	0	0	1	4	0.99	0.05
	Roy's Largest Root	0	0	1	4	0.99	0.05

Multivariate Design: Intercept+covariate+gender. Within Subjects Design: day

Table E2 Test of Within-Subjects Effects of Repeated Measures ANOVA of hemolymph osmolality data of days 5 and 9 of *L. chaiyaphumi* in 22ppt in shallow water and a covariate in 0ppt

Source		***Type I Sum of Squares***	***df***	***Mean Square***	***F***	***Sig.***	**Observed Power**
day	Sphericity Assumed	31114.29	1	31114.29	27.80	0.01 *	0.97
	Lower-bound	31114.29	1	31114.29	27.80	0.01 *	0.97
day* covariate	Sphericity Assumed	7793.55	1	7793.55	6.96	0.06	0.52
	Lower-bound	7793.55	1	7793.55	6.96	0.06	0.52
Day*gender	Sphericity Assumed	0.22	1	0.22	0	0.99	0.05
	Lower-bound	0.22	1	0.22	0	0.99	0.05
Error(day)	Sphericity Assumed	4476.94	4	1119.24			
	Lower-bound	4476.94	4	1119.24			

Table E3 Tests of Between-Subjects Effects of Repeated Measures ANOVA of hemolymph osmolality data of days 5 and 9 of *L. chaiyaphumi* in 22ppt in shallow water and a covariate in 0 ppt

Source	**Type I of Sum Squares**	**df**	**Mean Square**	**F**	**Sig.**	**Observed Power**
Intercept	4665623.143	1	4665623.14	1879.77	0.00 *	1.00
covariate	16662.18585	1	16662.19	6.71	0.06	0.50
gender	147.6085246	1	147.61	0.06	0.82	0.05
Error	9928.062765	4	2482.02			

Table E4 One-way ANOVA (estimated marginal means??) of Repeated Measures ANOVA of hemolymph osmolality data of days 5 and 9 of *L. chaiyaphumi* in 22ppt in shallow water and a covariate in 0 ppt

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	179584.03	2	89792.01	39.01	0.00 *
Within Groups	69059.49	30	2301.98		
Total	248643.52	32			

Table E5 Multiple comparisons Repeated Measures ANOVA of hemolymph osmolality data of days 5 and 9 of *L. chaiyaphumi* in 22ppt in shallow water and a covariate in 0 ppt

	(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	covariate	Day 5	-106.13	18.87	0.000 *	-152.66	-59.60
		Day 9	-188.14	22.21	0.000 *	-242.90	-133.39
	Day 5	covariate	106.13	18.87	0.000 *	59.60	152.66
		Day 9	-82.01	22.82	0.003 *	-138.27	-25.76
	Day 9	covariate	188.14	22.21	0.000 *	133.39	242.90
		Day 5	82.01	22.82	0.003 *	25.76	138.27
Dunnett t (2-sided)(a)	Day 5	covariate	106.13	18.87	0.000 *	62.10	150.17
	Day 9	covariate	188.14	22.21	0.000 *	136.33	239.96

* The mean difference is significant at the .05 level. a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Box's M test assumption of equality of covariance matrices was met (p = 0.880). The assumptions of sphericity could not be verified as there were not enough degrees of freedom. Levene's test assumption of equality of variances were met after 8 days (df = 13, p = 0.822) and 12 days (df = 13, p = 0.365).

Table E6 Multivariate Test (c) of Repeated measures ANOVA of hemolymph osmolality data of days 8 and 12 of juvenile and mini *L. chaiyaphumi* in shallow water with salinities of 0ppt, 22ppt and 30ppt and no gender differentiation

Effect		*Value*	*F*	*Hyp. df*	*Error df*	*Sig.*	*Observed Power*
Day	Pillai's Trace	0.02	0.29	1	13	0.60	0.08
	Roy's Largest Root	0.02	0.29	1	13	0.60	0.08
Day*gender	Pillai's Trace	0.30	5.55	1	13	0.03 *	0.59
	Roy's Largest Root	0.43	5.55	1	13	0.03 *	0.59
Day*salinity	Pillai's Trace	0.42	9.27	1	13	0.01 *	0.80
	Roy's Largest Root	0.71	9.27	1	13	0.01 *	0.80
Day*gender *salinity	Pillai's Trace	0.38	8.09	1	13	0.01 *	0.75
	Roy's Largest Root	0.62	8.09	1	13	0.01 *	0.75

Multivariate Design: Intercept+gender+salinity+gender * salinity. Within Subjects Design: day

Table E7 Test of Within-Subjects Effects of Repeated measures ANOVA of hemolymph osmolality data of days 8 and 12 of juvenile and mini *L. chaiyaphumi* in shallow water with salinities of 0ppt, 22ppt and 30ppt and no gender differentiation

Source		Type I Sum of Squares	df	Mean Square	F	Sig.	Observed Power
day	Sphericity Assumed	300.03	1	300.03	0.29	0.60	0.08
	Lower-bound	300.03	1	300.03	0.29	0.60	0.08
day*gender	Sphericity Assumed	5790.53	1	5790.53	5.55	0.03 *	0.59
	Lower-bound	5790.53	1	5790.53	5.55	0.03 *	0.59
day*salinity	Sphericity Assumed	9673.10	1	9673.10	9.27	0.01 *	0.80
	Lower-bound	9673.10	1	9673.10	9.27	0.01 *	0.80
day*gender* salinity	Sphericity Assumed	8433.13	1	8433.13	8.09	0.01 *	0.75
	Lower-bound	8433.13	1	8433.13	8.09	0.01 *	0.75
Error(day)	Sphericity Assumed	13558.71	13	1042.98			
	Lower-bound	13558.71	13	1042.98			

Table E8 Tests of Between-Subjects Effects of Repeated measures ANOVA of hemolymph osmolality data of days 8 and 12 of juvenile and mini *L. chaiyaphumi* in shallow water with salinities of 0ppt, 22ppt and 30ppt and no gender differentiation

Source	Type I Sum of Squares	df	Mean Square	F	Sig.	Observed Power
Intercept	7685506.62	1	7685506.62	3440.26	0.00 *	1
gender	3414.17	1	3414.17	1.53	0.238	0.21
salinity	104242.87	1	104242.87	46.66	0.00 *	1
gender*salinity	263.94	1	263.94	0.12	0.737	0.06
Error	29041.91	13	2233.99			

Table E9 Salinity Estimates of Repeated measures ANOVA of hemolymph osmolality data of days 8 and 12 of juvenile and mini *L. chaiyaphumi* in shallow water with salinities of 0ppt, 22ppt and 30ppt and no gender differentiation

salinity	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
.00	422.663	11.210	398.445	446.880
4.00	535.933	12.204	509.569	562.298

Table E10 Univariate Tests of Repeated measures ANOVA of hemolymph osmolality data of days 8 and 12 of juvenile and mini *L. chaiyaphumi* in shallow water with salinities of 0 ppt, 22 ppt and 30 ppt and no gender differentiation

	Sum of Squares	df	Mean Square	F	Sig.	Observed Power
Contrast	52190.976	1	52190.97	46.72	.000 *	1.00
Error	14520.954	13	1116.99			

Another Univariate Test: Levene's Test assumption of equality of variance was not met, instead a significant difference was found (F = 7.783, df1= 4, df2 = 36, p = 0.00).

Table E11 Test of Between-Subjects Effects (with dependent variable day 12) of Univariate ANOVA of hemolymph osmolality data of day 12 of juvenile and mini juvenile *L. chaiyaphumi* in different salinities (0, 22 and 30ppt), not including gender

Source	Type I Sum of Squares	df	Mean Square	F	Sig.	Observed Power
Corrected Model	514428.63	4	128607.16	28.09	0.00 *	1
Intercept	11054183.44	1	11054183.44	2414.21	0.00 *	1
salinity	508235.07	2	254117.53	55.50	0.00 *	1
age	1018.99	1	1018.99	0.22	0.64	0.07
salinity*age	5174.57	1	5174.57	1.13	0.29	0.18
Error	164836.93	36	4578.80			
Total	11733449.00	41				
Corrected Total	679265.56	40				

R Squared = .757 (Adjusted R Squared = .730)

Table E12 Univariate Test of Univariate ANOVA of hemolymph osmolality data of day 12 of juvenile and mini juvenile *L. chaiyaphumi* in different salinities (0, 22 and 30 ppt), not including gender

	Sum of Squares	df	Mean Square	F	Sig.	Observed Power(a)
Contrast	510895.17	2	255447.58	55.79	0.00 *	1
Error	164836.93	36	4578.80			

Table E13 Post-hoc Test of Univariate ANOVA of hemolymph osmolality data of day 12 of juvenile and mini juvenile *L. chaiyaphumi* in different salinities (0, 22 and 30 ppt), not including gender

	(I) salinity	*(J) salinity*	*Mean Difference (I-J)*	*Std. Error*	*Sig.*	*95% Confidence Interval*	
						Lower Bound	Upper Bound
Tukey HSD	0ppt	22ppt	-98.87	22.88	0.00 *	-154.81	-42.93
		30ppt	-338.15	32.13	0.00 *	-416.69	-259.61
	22ppt	0ppt	98.87	22.88	0.00 *	42.93	154.81
		30ppt	-239.28	31.90	0.00 *	-317.25	-161.31
	30ppt	0ppt	338.15	32.13	0.00 *	259.61	416.69
		22ppt	239.28	31.90	0.00 *	161.31	317.25
Bonferroni	0ppt	22ppt	-98.87	22.88	0.00 *	-156.33	-41.40
		30ppt	-338.15	32.13	0.00 *	-418.83	-257.46
	22ppt	0ppt	98.87	22.88	0.00 *	41.40	156.33
		30ppt	-239.28	31.90	0.00 *	-319.38	-159.18
	30ppt	0ppt	338.15	32.13	0.00 *	257.46	418.83
		22ppt	239.28	31.90	0.00 *	159.18	319.38
Dunnett t (2-sided)(a)	22ppt	0ppt	98.87	22.88	0.00 *	45.86	151.87
	30ppt	0ppt	338.15	32.13	0.00 *	263.72	412.57

Based on observed means. * The mean difference is significant at the .05 level. a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table E14 Variables not in the equation of the logistic regression of survival data of *L. chaiyaphumi* on day 9

			Score	df	Sig.
Step 0	Variables	salinity	1.22	1	0.27
		age	4.15	2	0.13
		age1 juvenile	0.01	1	0.93
		age2 adult	3.64	1	0.06
	Overall Statistics		4.82	3	0.19

Table E15 Variables not in the equation of the logistic regression of survival data of *L. chaiyaphumi* on day 13

			Score	df	Sig.
Step 0	Variables	salinity	1.33	1	0.25
		age	9.87	2	0.01 *
		age1 juvenile	6.81	1	0.01 *
		age2 adult	0.35	1	0.55
	Overall Statistics		12.04	3	0.01 *

Table E16 Relative risk of dying in *L. chaiyaphumi* in increased salinities compared to the control (fresh water) after 5, 9 and 14 days. The relative risk criterion is meaningful > 2 and < 0.5.

Water depth	Salinity in ppt	Gender / age	**DAY 5**	**DAY 9**	**DAY 14**
shallow	22	FA	**1.20**	**0.75**	**0.75**
shallow	22	MA	**1**	**0.67**	**1**
shallow	22	FJ	**1.20**	**1.05**	**0.75**
shallow	22	MJ	**0.92**	**0.42***	**0.50**
shallow	22	mini	**0.1***	**0.1***	**0.1***
shallow	30	mini	**2**	**3***	**3***

Table E17 Relative risk of dying in male compared to female *L. chaiyaphumi* in 22 ppt shallow water after 5, 9 and 14 days. The relative risk criterion is meaningful > 2 and < 0.5.

Salinity in ppt	**Water depth**	**Gender / age**	**Day 5**	**Day 9**	**Day 14**
22	shallow	MJ vs FJ	8.4*	1.95	1.34
22	shallow	MA vs FA	1	0.67	0.67

APPENDIX F

One-way ANOVA's

Table F1 Test of Homogeneity of Variances of day 5 osmolality data of species control groups

Levene Statistic	df1	df2	Sig.
2.515	2	76	.088

Table F2 Multiple comparisons Tukey HSD day 5 osmolality data of species control groups

(I) species	(J) species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-37.23	14.36	0.03 *	-71.55	-2.90
	3	-55.68	9.96	0.00 *	-79.49	-31.86
2	1	37.23	14.36	0.03 *	2.90	71.55
	3	-18.45	15.80	0.48	-56.21	19.31
3	1	55.68	9.96	0.00 *	31.86	79.49
	2	18.45	15.80	0.48	-19.31	56.21

Table F3Test of Homogeneity of Variances day 9 osmolality data of species control groups

Levene Statistic	df1	df2	Sig.
.858	3	77	.466

Table F4 Multiple Comparisons Tukey HSD day 9 osmolality data of species control groups

(I) species	(J) species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-4.52	14.77	0.99	-43.30	34.25
	3	-54.38	11.11	0.00 *	-83.57	-25.20
	4	-4.68	13.62	0.99	-40.43	31.08
2	1	4.52	14.77	0.99	-34.25	43.30
	3	-49.86	16.27	0.02 *	-92.58	-7.14
	4	-0.15	18.07	1.00	-47.60	47.30
3	1	54.38	11.11	0.00 *	25.20	83.57
	2	49.86	16.27	0.02 *	7.14	92.58
	4	49.71	15.23	0.01 *	9.71	89.71
4	1	4.68	13.62	0.99	-31.08	40.43
	2	0.15	18.07	1.00	-47.30	47.60
	3	-49.71	15.23	0.01 *	-89.71	-9.71

* The mean difference is significant at the .05 level.

The assumption of equal variances for the ANOVA was not fulfilled for the control day 13 test (p = 0.034).

Table F5 Test of Homogeneity of Variances day 13 of species control groups

Levene Statistic	df1	df2	Sig.
3.566	2	60	.034 *

Table F6 Multiple Comparisons Dependent Variable: day 13 Tukey HSD of species control groups

(I) species	(J) species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	3	-101.13	15.07	0.00 *	-137.36	-64.91
	4	-67.74	13.33	0.00 *	-99.78	-35.69
3	1	101.13	15.07	0.00 *	64.91	137.36
	4	33.40	16.92	0.13	-7.28	74.07
4	1	67.74	13.33	0.00 *	35.69	99.78
	3	-33.40	16.92	0.13	-74.07	7.28

* The mean difference is significant at the .05 level.

Table F7 Test of Homogeneity of Variances Day 9 of species 22ppt groups

Levene Statistic	df1	df2	Sig.
2.045	3	83	.114

Table F8 Multiple Comparisons Tukey HSD Day 9 of species 22ppt groups

(I) Species	(J) Species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-94.75	24.80	0.00 *	-159.77	-29.73
	3	-95.66	17.42	0.00 *	-141.32	-50.00
	4	-63.54	15.25	0.00 *	-103.51	-23.56
2	1	94.75	24.80	0.00 *	29.73	159.77
	3	-0.90	26.61	1.00	-70.68	68.87
	4	31.21	25.25	0.61	-34.98	97.41
3	1	95.66	17.42	0.00 *	50.00	141.32
	2	0.90	26.61	1.00	-68.87	70.68
	4	32.12	18.05	0.29	-15.21	79.44
4	1	63.54	15.25	0.00 *	23.56	103.51
	2	-31.21	25.25	0.61	-97.41	34.98
	3	-32.12	18.05	0.29	-79.44	15.21

* The mean difference is significant at the .05 level.

Table F9 Test of Homogeneity of Variances Day 13 of species 22ppt groups

Levene Statistic	df1	df2	Sig.
3.034	2	59	.056

Table F10 Multiple Comparisons Tukey HSD Day 13 of species 22ppt groups

(I) Species	(J) Species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	3	17.89	18.38	0.60	-26.29	62.07
	4	102.37	18.06	0.00 *	58.95	145.79
3	1	-17.89	18.38	0.60	-62.07	26.29
	4	84.48	20.07	0.00 *	36.22	132.74
4	1	-102.37	18.06	0.00 *	-145.79	-58.95
	3	-84.48	20.07	0.00 *	-132.74	-36.22

* The mean difference is significant at the .05 level.

Table F11 Test of Homogeneity of Variances Day 5 of species 30ppt groups

Levene Statistic	df1	df2	Sig.
21.903	2	93	.000 *

Table F12 Multiple Comparisons Dependent Variable: Day 5 Tukey HSD of species 30ppt

(I) species	**(J) species**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**	**95% Confidence Interval**	
					Lower Bound	Upper Bound
1	2	-6.92	18.92	0.93	-51.99	38.16
	3	40.30	16.91	0.05 *	0.03	80.57
2	1	6.92	18.92	0.93	-38.16	51.99
	3	47.21	20.82	0.07	-2.37	96.80
3	1	-40.30	16.91	0.05 *	-80.57	-0.03
	2	-47.21	20.82	0.07	-96.80	2.37

* The mean difference is significant at the .05 level.

Table F13 Test of Homogeneity of Variances Day 9 of species 30ppt group

Levene Statistic	df1	df2	Sig.
5.534	2	58	.006 *

Table F14 Multiple Comparisons Day 9 of species 30 ppt group

(I) species	(J) species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-41.30	20.58	0.12	-90.80	8.20
	3	15.65	18.43	0.67	-28.69	59.99
2	1	41.30	20.58	0.12	-8.20	90.80
	3	56.95	21.77	0.03 *	4.58	109.32
3	1	-15.65	18.43	0.67	-59.99	28.69
	2	-56.95	21.77	0.03 *	-109.32	-4.58

* The mean difference is significant at the .05 level.

Table F15 Test of Homogeneity of Variances Day 13 of species group 30 ppt

Levene Statistic	df1	df2	Sig.
12.604	2	47	.000 *

Table F16 Multiple Comparisons Day 13 of species 30 ppt group

(I) species	(J) species	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	3	62.00	20.83	0.01 *	11.58	112.41
	4	19.38	30.77	0.80	-55.08	93.85
3	1	-62.00	20.83	0.01 *	-112.41	-11.58
	4	-42.61	32.03	0.39	-120.12	34.90
4	1	-19.38	30.77	0.80	-93.85	55.08
	3	42.61	32.03	0.39	-34.90	120.12

* The mean difference is significant at the .05 level.

Table F17 Relative risk of dying in fresh water of *S. germaini, P. smithianum* and *L. chaiyaphumi* compared to *E. dugasti.* The relative risk criterion is meaningful > 2 and < 0.5.

species	**groups**		**Day 5**	**Day 9**	**Day 14**
S. germaini vs *E. dugasti*	shallow	FA	**1**	**0.02***	
S. germaini vs *E. dugasti*	shallow	MA	**0.05***	**0.03***	
P. smithianum vs *E. dugasti*	shallow	FA	**20***	**0.48***	**0.53**
P. smithianum vs *E. dugasti*	shallow	MA	**1.50**	**0.90**	**1**
P. smithianum vs *E. dugasti*	shallow	FJ	**5.20***	**7.80***	
P. smithianum vs *E. dugasti*	shallow	MJ	**60***	**60***	
L. chaiyaphumi vs *E. dugasti*	shallow	FJ	**2.17***	**4.34***	**1.09**
L. chaiyaphumi vs *E. dugasti*	shallow	MJ	**1**	**1**	**0.62**

Table F18 Relative risk of dying for *S. germaini, P. smithianum* and *L. chaiyaphumi* compared to *E. dugasti* after 5, 9 and 14 days in sea water of 28 and 30 ppt. The relative risk criterion is meaningful > 2 and < 0.5.

Salinity	species	Water depth	gender	**Day 5**	**Day 9**	**Day 14**
30 ppt	*S. germaini* vs *E. dugasti*	deep	FA	0.11*	0.26*	
30 ppt	*S. germaini* vs *E. dugasti*	deep	MA	0.05*	0.42*	
30 ppt	*P. smithianum* vs *E. dugasti*	deep	FA	4.4*	0.52	
30 ppt	*P. smithianum* vs *E. dugasti*	deep	MA	2.50*	0.70	
30 ppt	*L. chaiyaphumi* vs *E. dugasti*	shallow	mini vs FJ	0.87	1.30	0.65
28 ppt	*P. smithianum* vs *E. dugasti*	shallow	FA	0.12*	0.23*	0.27*