Title
Factors Affecting Sperm Motility, Fertilization and Early Development in the Pacific Herring (Clupea pallasi)

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Publication Date
1998

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Factors Affecting Sperm Motility, Fertilization and Early Development in the Pacific Herring (*Clupea pallasi*)

By

CAROL ANN VINES
B.S. (California State University, San Francisco) 1968

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

Pharmacology and Toxicology

in the

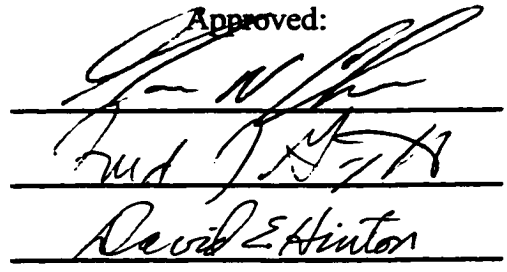
OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

Davis

Approved:



Committee in Charge

1998

TABLE OF CONTENTS

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March 1999
Pharmacology and Toxicology

Factors Affecting Sperm Motility, Fertilization and Early Development in the Pacific Herring (*Clupea pallasi*)

Abstract

The effects of an environmental stressor (salinity) and an anthropogenic stressor (creosote-derived compounds) on sperm motility, fertilization and early development were investigated in the Pacific herring. In the laboratory, the optimal salinity for sperm motility initiation in response to an egg-derived factor, sperm motility initiating factor (SMIF), was 16-24 parts per thousand (ppt), while the optimal salinity for both fertilization and development was 12-24 ppt. These salinities are approximately one-half that of full-strength seawater. These laboratory data correlated with the optimal salinity for development (16-20 ppt) for embryos deployed at field sites in San Francisco Bay. The effect of individual cations on fertilization was also assessed. Sodium (Na^+) inhibited fertilization at concentrations $\leq$55 or $\geq$440 mM. Alterations in calcium or magnesium had no effect on fertilization unless omitted from the medium. A similar effect was seen for potassium (K^+), but elevated K^+ in combination with elevated Na^+ inhibited fertilization to a greater degree than Na^+ alone.

To characterize the mechanism for the effects of altered salinity on sperm motility, changes in intracellular ions in response to SMIF or by manipulation of extracellular ions were investigated. SMIF induced a membrane depolarization that was inhibited by increasing either salinity or the Na^+ concentration. SMIF, and an activator of

protein kinase C, induced an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), which along with motility initiation required the presence of extracellular Ca^{2+} ($[Ca^{2+}]_o$). An efflux of Na^+ was observed in response to SMIF, or in the absence of SMIF, in low sodium media, again requiring $[Ca^{2+}]_o$. Membrane depolarization, increase in $[Ca^{2+}]_i$, and Na^+ efflux were inhibited by calcium channel and sodium-calcium exchange inhibitors.

Finally, the effects of creosote-derived compounds on development were investigated. Exposed embryos exhibited significant mortality, delay in development, cardiac abnormalities, abnormal movement within the chorion, and edema. Hatching success of exposed embryos was also decreased, and all larvae exhibited severe morphological deformities (scoliosis, pericardial and/or yolk-sac edema. Less than optimal salinities (8 and 28 ppt) enhanced the effects of creosote-derived compounds on development.

Introduction

The Pacific herring (*Clupea pallasi*) is a marine teleost fish belonging to the Clupeoidea, a group which including sardines, anchovies and pilchards, comprise about one third of the world's fisheries (reviewed by Blaxter and Hunter, 1982). Pacific herring constitute the third largest fishery in California, with San Francisco Bay supporting the largest commercial catch (California Department of Fish and Game, 1995). Although a small fishery for bait and animal feed operates in northern California during the summer and fall, the majority of herring are harvested for sac-roe during the spawning season (winter-spring). A smaller roe on kelp fishery also operates in San Francisco Bay during this period. The majority of roe or roe on kelp harvested in California are exported to Japan, where they are considered to be delicacies. In addition to their commercial importance, all life stages of herring represent an important source of food for other marine and estuarine organisms, including birds, other fish (salmon, sturgeon), and marine mammals (Alderdice and Hourston, 1985; California Department of Fish and Game, 1995). Herring occupy marine waters during the non-reproductive season, but migrate to and spawn in bays and estuaries in winter-spring (Alderdice and Velsen, 1971; Alderdice and Hourston, 1985). In Northern California, Pacific herring spawning may occur from November to March in San Francisco Bay and Tomales Bay (Rabin and Barnhart, 1986; Reilly *et al.*, 1989), presumably to take advantage of the peak in plankton production (Blaxter and Hunter, 1982; Haegele and Schweigert, 1985). With increasing latitude, the onset of spawning occurs later in the spring and for a shorter period of time; Pacific herring in British Columbia, for example, spawn in March and April (Hay, 1990). Herring tend to spawn in waves, with the older age-classes spawning

first, followed by younger fish later in the season (California Department of Fish and Game, 1995). Males typically spawn first, and this stimulates females as well as other males to also spawn (Stacey and Hourston, 1982), and there is evidence suggesting that milt contains a pheromone-like substance that stimulates spawning by other herring (Sherwood *et al.*, 1991). Herring eggs adhere to a variety of substrates, including marine vegetation, rocks, pilings, and shallow gravel bottoms and embryonic development through hatching takes place upon these substrates (Haegele *et al.*, 1981). Time to hatch is dependent on latitude and temperature and ranges from 9 days in Northern California to as many as 30 days in Alaska.

Spawning in bays and estuaries during the winter months also coincides with an increased input of freshwater during normal rainfall years. Overall, salinities on the spawning grounds range from 5-35 ppt (Holliday and Blaxter, 1960; Haegele and Schweigert, 1985), however, the optimal salinity for reproduction in individual populations probably encompasses a much narrower range, and the selection of spawning grounds likely reflects the tolerance of individual populations for particular salinity ranges. For example, specific populations of herring in Japanese waters consistently spawn at salinities approaching those of normal seawater, while others spawn in lower salinity seawater (Kijima *et al.*, 1992; Kobayashi 1993). In Northern California (e.g. San Francisco and Tomales Bays), herring spawn in bays and estuaries having lowered salinities due to freshwater input during the winter and spring, and the optimal salinities for fertilization and early development are 12-24 ppt and 8-24 ppt respectively (Griffin *et al.*, 1998). The tendency of spawning herring to hold in these bays for days to weeks prior to spawning (Haegele and Schweigert, 1985), coupled with evidence that reduced

salinities may stimulate spawning in the field (Rabin and Barnhart, 1986), and in the lab (Outram, 1951) suggests that changes in salinity may be at least one of the factors regulating spawning behavior. In fact, drought conditions during the late 1980's and early 1990's, resulting in salinities approaching those of oceanic waters (averaging greater than 30 ppt), coincided with a dramatic decline in herring spawning biomass and recruitment in San Francisco Bay (California Department of Fish and Game, 1992). With the return of normal rainfall years (1995–1997), spawning biomass increased substantially. Differences in salinity tolerances also appear to be true for the Atlantic herring, *Clupea harengus* (Blaxter and Holliday, 1963; Ojaveer, 1981; Haegele and Schweigert, 1985). Although most populations of Atlantic herring are considered to be oceanic spawners, the optimal salinity for fertilization in the Baltic herring (*C. harengus membras*), a subspecies of Atlantic herring, is 8-16 ppt (Griffin *et al.*, 1998), most likely reflecting the fact that this population resides in reduced salinity seawater (4-8 ppt) throughout the year (Haapala and Alenius, 1994). The effect of salinity and specific ions on sperm motility, fertilization and development in Pacific herring from San Francisco Bay is the focus of chapter one. In the laboratory, fertilization and development assays were conducted at varying salinities utilizing Pacific herring gametes from fish collected in San Francisco Bay, as well as gametes from Baltic herring. Pacific herring embryos were also deployed at field sites in San Francisco Bay to determine the salinity tolerance range for development under field conditions. In addition, the effects of salinity on sperm motility initiation in Pacific herring was assessed, and sperm motility in Baltic herring was compared to that of Pacific herring.

Yanagimachi and Kanoh in the 1950's observed that herring sperm remain immotile upon release from the testis in salinities ranging from 8-35 ppt (Yanagimachi and Kanoh, 1953; Yanagimachi, 1956, 1957). So far as is known, this is unique in a teleost, but has been observed in the horseshoe crab (*Limulus*), in which sperm are immotile at spawning and initiate motility during gamete interaction (Clapper and Brown, 1980; Clapper and Epel, 1982), and in the ascidian, *Ciona* (Yoshida, 1995). They also observed that sperm motility is initiated upon contact with the micropyle region of the herring chorion, and that continued motility is dependent on repeated contact with this region. An egg-derived protein, sperm motility initiating factor (SMIF) was subsequently isolated and characterized, and found to initiate motility *in vivo* and *in vitro* (Pillai, *et al.*, 1993; Griffin *et al.*, 1996). SMIF is a 105 kDa, non-diffusible glycoprotein that is localized to the region of the micropyle, a region of the chorion containing the micropylar canal through which the fertilizing sperm must travel in order to fuse with the egg plasma membrane (Amanze and Iyengar, 1990). Both sperm motility and fertilization can be inhibited by treatment of eggs with anti-SMIF antibody. Isolated SMIF appears to bind to the sperm surface in the midpiece region overlying the mitochondria (Griffin *et al.*, 1996), suggesting an interaction between SMIF and a receptor, resulting in initiation of sperm motility. Morisawa and colleagues have also isolated and characterized several low molecular weight peptides (herring sperm activating peptides or HSAP's), which also appear to initiate sperm motility (Morisawa *et al.*, 1992; Oda *et al.*, 1995). The relationship, if any, between SMIF and HSAP's remains to be clarified.

Herring sperm motility is also affected by salinity. SMIF induced motility is inhibited both in reduced (4 ppt) and elevated (32 ppt) salinities, with the optimal salinity

for initiation ranging from 16-24 ppt (Griffin *et al.*, 1998). The reduction in sperm motility initiation at both reduced and elevated salinities correlates with the reduction in fertilization rates observed at these same salinities. Further, it has been shown that herring sperm maintain the ability to fertilize longer in reduced salinity seawater (Yanagimachi and Kanoh, 1953). Motility initiation in the micropyle region is dependent on the presence of calcium (Ca^{2+}) and potassium (K^+) (Yanagimachi *et al.*, 1992), however the mechanism for this dependency is not known. Motility can be artificially initiated in the absence of SMIF using low sodium (Na^+) seawater (3 mM Na^+), suggesting that an efflux of sodium from the sperm may be important in motility initiation, at least under these conditions. The fact that fertilization is also inhibited with increasing concentrations of Na^+ and K^+ (Griffin *et al.*, 1998), suggests that the reduction in fertilization observed at elevated salinities is due to impairment of motility initiation at elevated levels of these ions. At lower salinities, the reduction in fertilization may be related to less than optimal concentrations of Ca^{2+} or K^+ ions. Chapter two will focus on changes in intracellular ions associated with sperm motility initiation, primarily in response to SMIF, as well as the effect of alterations in extracellular ions on this process. Motility initiation using low Na^+ seawater (in the absence of SMIF) will also be investigated.

Ever increasing human demands on estuaries such as San Francisco Bay for residential, recreational, and commercial purposes have profound impacts on estuarine organisms in the form of increased pollution, habitat destruction, physical disturbance, competition for resources, or overfishing (San Francisco Estuary Report, 1991; Nichols, 1995). Reproduction in estuarine organisms can be perturbed in several ways. Removal

of adults through disease, predation or fishing also results in the loss of gametes available for fertilization. Gametes and the early developmental stages of organisms are especially vulnerable to alterations in their environment, (Rand and Petrocelli, 1985, von Westernhagen, 1988, Manson and Wise, 1991). This is particularly true for the many aquatic organisms that release their gametes into the environment, and/or whose embryonic and larval stages develop externally in the absence of parental care. Embryo mortality or malformations are often observed at lower concentrations than those that would affect adult organisms (Bantle, 1985; Von Westernhagen, 1988; Manson and Wise. 1991). In addition, key developmental events may be targets for pollutant effects. Normal gene expression or repression during critical developmental periods may be targets for toxic agents. The rapid cell division and differentiation characteristic of early development may not allow affected cells or tissues to undertake effective repair of DNA damage. Toxic agents may interfere with cell signaling events required for differentiation of cells or tissues, or with migration of cells to form the precursors of adult tissues. Embryos or larvae may lack detoxification mechanisms to neutralize particular agents. Finally, the metabolic costs of DNA repair or detoxification may be too great for particular stages.

Alterations in salinity due to anthropogenic (water diversion) or natural causes (drought) are only one type of stressor that may affect reproduction in Pacific herring. However, other factors present in estuarine environments may also impact reproduction, acting individually or in combination with other stressors. In recent years, the loss of *Gracilaria* beds, a favored substrate for spawning, has led to a shift in spawning substrate preferences (Spratt, 1992). As a consequence, herring spawning onto less than optimal

substrates, such as marine pilings, has increased. Much of the San Francisco waterfront contains creosote-treated pilings upon which herring have been observed to spawn (Diana Watters, personal communication). Since herring embryos remain attached to substrates for several days during incubation, they are particularly vulnerable to the effects of toxicants in their immediate environment. The toxic effects of creosote, a wood preservative, have been demonstrated in a number of organisms both in the laboratory and in the field (Von Burg and Stout, 1992; Malins *et al.*, 1985; Fournie and Vogelbein, 1994a,b; Gagne *et al.*, 1995; Shugart, 1995). However, only limited studies on the effects of creosote on early reproductive events have been performed (Peterson *et al.*, 1978; Iyer et al. 1992, 1993), particularly on organisms in the marine environment. Chapter three focuses on the effects of creosote-treated wood on early development through hatching in the Pacific herring. The combined affects of creosote and salinity are also investigated.

The commercially and ecologically important Pacific herring provides a model system for investigating a variety of biological questions. Chapter one expands previous investigations of salinity effects on fertilization and early development. Chapter two investigates the ionic conditions and mechanisms by which motility initiation takes place. Chapter three investigates perturbation of development due to exposure to creosote, alone and in combination with salinity. It is hoped that further elucidation of the basic biology of herring reproduction will offer a basis for evaluating policies and management issues important in the future of San Francisco Bay.

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CHAPTER 1

The Effects of Salinity on Sperm Motility, Fertilization and Early Development in Herring

Abstract

The effects of salinity on sperm motility, fertilization and early development in the Pacific herring (*Clupea pallasi*) from San Francisco Bay, CA were investigated. This population experiences a wide range of salinities, residing in marine waters with salinities >30 parts per thousand (ppt) for most of the year, but spawning in an estuarine environment with salinities of 12-20 ppt in normal rainfall years. Baltic herring (*Clupea harengus membras*), which reside and spawn in lowered salinities (4-8 ppt) in Airisto Sound, Finland, were used to compare the effects of adult salinity exposure on the salinity tolerance range for fertilization and development. The salinity tolerance range for fertilization in Pacific herring fell between 8 and 28 ppt, with an optimal range of 12 to 24 ppt, while that for the Baltic herring was shifted downward to 4-24 ppt, with an optimal range of 8-16 ppt. Salinity tolerance ranges for embryonic development for both species were similar to those for fertilization; the Pacific herring exhibited a range of 8-24 ppt, while that for Baltic herring ranged from 4-20 ppt. Cross-fertilization of Baltic herring eggs with Pacific herring sperm resulted in salinity tolerance ranges for fertilization and development of 8-20 ppt and 8-12 ppt respectively. The salinity tolerance range for development in Pacific herring embryos in the laboratory was similar to that observed in embryos deployed at two field sites. The effect of major cations found in seawater on fertilization in the Pacific herring was also investigated. Sodium (Na^+) had the greatest effect, inhibiting fertilization at concentrations $\leq$55mM and $\geq$ 440 mM. Increasing the concentrations of potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) had no effect on fertilization, but complete absence of K^+ or Ca^{2+}, and to a lesser degree,

Mg^{2+}, resulted in inhibition of fertilization. While increasing K^+ alone had no effect on fertilization, a synergistic effect with Na^+ was observed, such that a lower concentration of Na^+ (330 mM) was required to inhibit fertilization when 10 mM K^+ was present, as compared to the concentration required (440 mM) to inhibit fertilization when 5 mM K^+ was present. Pacific herring sperm are unique in that they are immotile at spawning, but initiate motility in response to sperm motility initiating factor (SMIF) an egg-derived glycoprotein (Pillai *et al.*, 1993; Griffin *et al.*, 1996). Sperm motility initiation in response to SMIF was optimal in 16-24 ppt salinities, and was markedly decreased at salinities $\leq$12 ppt and $\geq$28 ppt. Sperm motility initiation was also investigated in the Baltic herring and found to be similar to that for the Pacific herring. Baltic sperm were immotile in seawater, and initiated motility in response to Pacific SMIF and to an egg-derived protein isolated from Baltic eggs. This protein (105 kDa) co-migrated with SMIF and was recognized by an antibody specific for Pacific herring SMIF. Baltic sperm also initiated motility in low Na^+ seawater (NaF), previously found to initiate motility in the absence of SMIF in Pacific herring (Yanagimachi *et al.*, 1992).

Introduction

Pacific herring, *Clupea pallasi*, reside in offshore waters over the continental shelves of the North Pacific Ocean from California to Korea, and in the White and Kara Sea regions of the Arctic Ocean (Dushkina, 1973; Grant, 1984; Grant and Utter, 1984), but migrate to shallower, near-shore waters during the winter and spring to spawn (Haegele and Schweigert, 1985). Pacific herring were once considered to be a subspecies of the Atlantic herring (*Clupea harengus*), as is the Baltic herring, but are now recognized to be different behaviorally, morphologically, and genetically from *C. harengus* (Grant, 1984; Grant and Utter, 1984; Haegele and Schweigert, 1985). Pacific herring spawn during the winter and spring months, migrating to shallower, near-shore waters of estuaries and bays. Males typically spawn first, triggering other males and females to begin spawning (Hourston, *et al.*, 1977; Stacey and Hourston, 1982). Males release sperm into the water, while females deposit adhesive eggs onto a variety of intertidal and subtidal substrates, and development through hatching takes place upon these substrates (Haegele and Schweigert, 1985). Unlike the sperm of most other species, herring sperm are not motile at the time of spawning, but initiate motility upon contact with an egg-derived factor termed sperm motility initiating factor, or SMIF (Yanagimachi and Kanoh, 1953; Pillai *et al.*, 1993).

Laboratory studies have shown that salinity has an influence on spawning, sperm motility, fertilization, and embryonic development in the Pacific herring (Alderdice and Hourston 1985; Taylor, 1971; Yanagimachi *et al.*, 1992; Pillai *et al.*, 1993; Griffin et

al. 1998). However, the importance of salinity has been questioned in light of the broad range of salinities (5-35 ppt) observed at spawning sites known to be utilized by Pacific herring (Blaxter and Holliday, 1963; Haegele and Schweigert, 1985). This broad range of salinities may reflect a composite of narrower ranges specific to distinct stocks or populations of herring, rather than an overall tolerance of the species as a whole.

It has previously been shown that certain cations influence herring sperm motility (Yanagimachi, 1953; Yanagimachi and Kanoh 1953; Yanagimachi, 1957; Yanagimachi *et al.*, 1992). *In vivo*, both extracellular K^+ and Ca^{2+} are required for motility initiation in the micropyle region of the herring egg, while extracellular Na^+ is not required (Yanagimachi and Kanoh, 1953; Yanagimachi *et al.*, 1992). *In vitro*, low Na^+ seawater can initiate motility in the absence of SMIF, while media lacking Ca^{2+} or K^+ does not have a similar effect. The mechanisms by which specific ions affect motility, however, remain to be determined.

Pacific herring in the San Francisco estuary constitute the southernmost spawning population and are known to be impacted by fluctuations in climate that impact freshwater input, and thus salinity in the bay; i.e. dry years (drought) and very wet years (El Nino). This study was undertaken to investigate the tolerance of gametes and embryos to salinities that may be encountered in the estuary. Due to the variability in freshwater input from year to year, the salinity exposure history of adult herring is not known for certain. In addition, it is not known if herring in San Francisco Bay constitute a single population, or discrete populations with different salinity exposure histories prior to spawning. Thus, the salinity tolerance range for fertilization and development in Pacific herring from San Francisco Bay was compared to a population of Baltic herring

known to reside and spawn in a low salinity environment (4-8 ppt) that does not fluctuate from year to year (Haapala and Alenius, 1994). Cross-fertilization experiments using Baltic herring eggs fertilized with Pacific herring sperm were also performed and the salinity tolerance range determined for these hybrids. Partial characterization of sperm motility initiation in the Baltic herring was also investigated, and compared to motility initiation in the Pacific herring. In addition to overall salinity effects on Pacific herring, the role of specific ions in the fertilization process was investigated. Finally, the salinity tolerance range of embryos deployed at field sites with differing salinity profiles was evaluated and compared to that observed in laboratory experiments.

Materials and Methods

Collection of animals and gametes

Pacific herring were collected by otter trawl from the San Francisco Bay Estuary in collaboration with biologists from the California Department of Fish and Game, and transported to the Bodega Marine Laboratory at 4°C within 4 hours of collection. Testes and ovaries were dissected as previously described (Griffin *et al.*, 1998), and stored at 4°C under moist conditions. Gametes from Pacific herring gonads stored under these conditions maintain their viability for several days.

Baltic herring were collected from Airisto Sound (Turku, Finland) by researchers from University of Turku, Finland. Gonads were dissected and placed in individual petri plates under moist conditions at 4°C, and shipped within 18 hours to the Bodega Marine Laboratory.

Sperm for motility, fertilization, and development assays were suspended in Herring Ringers consisting of 206 mM NaCl, 7.2 mM KCl, 2.1 mM $CaCl_2$, 3.1 mM $MgCl_2 \cdot 6H_2O$, pH adjusted to 7.8 using 1M $NaHCO_3$ (Yanagimachi, 1957).

Filtered seawater (FSW) was prepared by filtering raw seawater through a 0.45 mm Nalgene filter (Fisher Scientific, Pittsburgh, PA). 4, 8 (1/4 FSW), 12, 16 (1/2 FSW), 20, 24, 28, and 32 parts per thousand (ppt) seawaters were prepared by diluting FSW with distilled water (dH_2O). Artificial seawaters (ASW) were prepared according to Cavanaugh (1975), substituting choline chloride for Na^+ or K^+ in low Na^+ and K^+ free seawaters, and adding 5 mM EDTA or EGTA for Ca^{2+} and Mg^{2+} free seawaters respectively. ½ ASW's containing varying concentrations of Na^+, K^+, Ca^{2+}, or Mg^{2+} were prepared as follows: for ion concentrations less than those found in ½ ASW, ½ ASW lacking the ion was mixed with ½ ASW containing twice the concentration of the ion of interest; for ion concentrations greater than those in ½ ASW, the appropriate volume of NaCl, KCl, $CaCl_2$, or $MgCl_2$ (as 5 M stock solutions in dH_2O) was added to ½ ASW. All seawaters were stored at 13°C. All dry chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Isolation of SMIF

Herring chorions were isolated according to Griffin et al. (1996). Herring eggs were suspended in1/2 CaMgF with 0.25% PVA at 4°C and washed 3-5 times to remove ovarian fluid. Eggs were disrupted in a tissue homogenizer 2-3 times in 1/2 CaMgF, and the isolated chorions were washed extensively in 1/2 CaMgF. Isolated chorions were either used immediately or rinsed in dH_2O, lyophilized, and stored at -70°C for further use. SMIF was isolated as previously described (Pillai *et al.*, 1993). Briefly, isolated

chorions were suspended in1/2 ASW (pH 3.5) at 4°C for 30 minutes, with periodic homogenization. The resulting homogenate was adjusted to pH 7.8 and centrifuged at 12,000 g for 15 min. The supernatant was concentrated using 10 kDa MW Centricon 10 microconcentrators (Amicon Co., Beverly, MA). The retentate was aliquoted and used immediately or stored at -70°C for further use. Prior to use of SMIF in experiments, the highest dilution which yielded 50-75% motility (3-4+ motility score; see below) was determined and used for all subsequent experiments.

Assessment of Sperm Motility:

Sperm motility was assessed as previously described (Griffin *et al.*, 1996). Twenty µl of ½ low sodium ("sodium-free") seawater (1/2 NaF) or ½ FSW were placed in individual wells of multi-well immunoslides (Polysciences, Inc., PA) and 1 µl of sperm (10^6 sperm/ml) suspended in HR was added to each well. Motility was scored using darkfield optics and a 20x objective lens on a compound microscope, and evaluated using the following qualitative index: 0 = no motility, 1 = up to 25% motility, 2 = 25-50% motility, 3 = 50-75% motility, and 4 = >75% motility. Sperm exhibiting 3-4+ motility (>75% motility) in response to ½ NaF, with no motility observed in ½ FSW were selected for fertilization assays.

To investigate initiation of Baltic herring sperm motility in response to Baltic herring SMIF and Pacific herring SMIF, 20 µl of SMIF diluted in ½ ASW (50-100 µg protein SMIF/ml) were placed into individual wells of immunoslides, and 1 µl of sperm (10^6 sperm/ml) added to each well. Motility was scored as above.

Electrophoresis and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of Pacific and Baltic SMIF was performed on 4-15% minigels (BioRad Laboratories, Hercules, CA). Samples were diluted in 2x solubilization buffer (Laemmli, 1970) containing 0.5 M Tris-HCl (pH 6.8), 10% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, 22% glycerol, and dH_2O and heated for 5 min. at 90°C. Proteins were visualized by staining the gels with Coomassie blue. For further characterization, the 105 kDa band was excised from the gel, electroluted, and dialyzed overnight against dH_2O (Pillai *et al.*, 1994). For immunoblotting, proteins were isolated on SDS-PAGE, then electrophoretically transferred to nitrocellulose membranes (BioRad). Following immunoblotting, blots were blocked overnight in blocking solution containing Tris Buffered Saline (TBS) pH 7.4, containing 0.2% tween-20 (TBST) and 9% non-fat milk. Blots were incubated in anti-SMIF antibody (Antibodies Inc., Davis, CA) for 1 hour, then washed 4x in TBST (10 min each). Blots were then incubated in goat anti-rabbit IgG conjugated to horseradish peroxidase for 1 hour. Blots were washed in TBST 4x (10 min. each) and visualized using enhanced chemiluminescence (Amersham, MA).

Fertilization and development assays:

Fertilization assays were performed as per Griffin *et al.*, (1998). Sperm concentrations resulting in 70-90% fertilization were determined for individual Pacific herring males in 1/2 FSW (16 ppt), the optimal salinity for fertilization in this species (Yanagimachi et al. 1992) and ¼ FSW (8 ppt) for Baltic herring, based on salinities of 6-7 ppt in Airisto Sound, Finland (Vuorinen and Ranta, 1987). Sperm dilutions were incubated for 10 minutes in polystyrene culture dishes in 10 ml of the respective

seawaters, followed by the addition of eggs (~100) into the dishes. Eggs and sperm were incubated for 20 min, then rinsed with seawater to remove excess sperm. Successful fertilization was assessed by scoring the number of eggs with a clearly defined perivitelline space between chorion and egg (Pillai *et al.*, 1994).

For fertilization assays in varying salinities or in seawaters with varying concentrations of Na^+, K^+, Ca^{2+}, or Mg^{2+}, sperm were incubated in the respective media for 10 minutes, followed by the addition of eggs (~100) into the plates. Eggs and sperm were incubated for 20 minutes, rinsed to remove excess sperm, and fertilization assessed as above.

For assessing the effects of altered salinities on embryonic development and hatching, Pacific and Baltic eggs were fertilized in ¼ or ½ FSW respectively and incubated in varying salinity seawaters as described above. Embryos were incubated at 13°C with daily water changes until hatching. Embryonic development was monitored using an Olympus stereo-zoom microscope, and was assessed using terminology and stages described by Kimmel et al. (1995) for zebrafish; first cleavage, gastrulation, formation of somites, optic vesicle formation, and retinal pigmentation. Hatching was scored by counting the number of empty chorions attached to the dish versus the number of chorions containing unhatched embryos, and was assessed when greater than 40% of embryos in the ½ FSW controls had hatched. Larvae were assessed for spinal abnormalities (scoliosis) and mortality after hatching.

Cross fertilization experiments at varying salinities were performed using Baltic herring eggs and cryopreserved Pacific herring sperm, previously shown to retain fertilizing capability (Pillai *et al.*, 1994). Eggs were fertilized, and the fertilization

success determined as described above. Embryos were incubated at 13°C with daily water changes until hatching, and development and hatching success evaluated as described above.

Field Exposures

Salinity outplant experiments were conducted at two field sites, chosen for their differing salinity profiles and historical record as herring spawning sites. The first, Paradise Cove experiences significant freshwater input from the Sacramento and San Joaquin River drainages during the winter, and was selected on the basis of its lower salinity profile. The second, the Presidio Yacht harbor at Fort Baker, just inside the Golden Gate Bridge, was selected for its higher salinity profile due to its proximity to the open ocean. Salinity and temperature were measured on a YSI Model 33 S-C-T Meter(YSI Inc., Yellow Springs, OH) and accompanying water samples were collected on each outplant expedition. The apparatus selected for embryo exposure in the field consisted of chambers constructed of 11 cm PVC pipe with 253 μm nitex mesh (Tetko, OH) secured over each end to allow water exchange through the chamber. 100 eggs were deposited onto 2 x 8 cm sections of nitex mesh (253 μm) and fertilized as described above. Individual sections of the nitex mesh were placed into 50,000 MW cutoff Spectra/Por 7 membranes (Spectrum, , Houston, TX), previously shown to allow equilibration of salinities at the rate of 1 ppt/hour, filled with ½ FSW, and sealed. Embryos were transported to the field sites in ½ FSW at 13°C. The membranes were placed into the PVC chambers, and the chambers were attached to a 3/4 inch nylon rope secured to a pier and lowered into the water column. To ensure that chambers remained suspended in the

water column during incubation, the other end of the rope was attached to a 25-pound mushroom anchor. Embryos were outplanted at the sites within 4 hours of fertilization and were collected from each site ~1 day prior to hatching. Following collection, embryos were transported to the Bodega Bay Marine Laboratory in ambient seawater within 4 hours of collection. Upon arrival at the laboratory, the salinity of the transporting seawater was measured, as well as the medium within each membrane. The nitex mesh strips (with embryos attached) were removed from the membranes and incubated in finger bowls containing seawaters corresponding to the measured salinity of their respective membranes. Hatching success and larval morphology were assessed as described above.

Water samples collected from each site were analyzed for major cations (Na^+, K^+, Ca^{2+}, and Mg^{2+}) at the UC Veterinary Diagnostic Laboratory.

Statistical analysis

Experiments were conducted using gametes from individual females and males. Except for the cross-fertilization studies, three or more separate experiments with 3 replicates per exposure were set up. Mean values and one standard deviation around the mean were calculated for each salinity exposure, and analyzed using one way analysis of variance (ANOVA) and Tukeys's Pairwise Multiple Comparison test, or student's t test (Sigma Stat, Jandel Scientific, San Rafael, CA).

Results:

Baltic sperm motility

Baltic herring sperm had similar motility characteristics as previously described for Pacific herring sperm (Yanagimachi *et al.*, 1992). Baltic sperm were not motile in 1/4 or

1/2 FSW, but motility was initiated in response to 1/4 NaF. Motility in Baltic herring sperm was also initiated by contact with Pacific herring SMIF, as well as contact with an egg-derived protein isolated from Baltic chorions, presumed to be SMIF (see electrophoresis and immunoblotting below).

Electrophoresis and blotting:

Using a similar procedure to isolate Pacific SMIF (see above), an egg-derived protein was isolated from Baltic chorions that initiated motility in Baltic sperm. SDS-PAGE of this protein resulted in a band migrating at 105 kDa, similar to that previously observed with Pacific SMIF (Fig. 1). On immunoblots, this band was recognized by an antibody previously generated to Pacific herring SMIF, producing a strong signal at 105 kDa.

Effects of salinity on fertilization and development in Pacific and Baltic herring

The optimal salinity for fertilization in the Pacific and Baltic herring differ, but the range of salinities over which fertilization takes place overlaps. The optimal salinity for fertilization in the Pacific herring was 16 ppt (86% fertilization), with a significant decrease ($p<0.001$) at 8 ppt (80% decrease) and at 32 ppt (88% decrease) (Fig. 2). The optimal salinity for fertilization in the Baltic herring was shifted downward to 8 ppt (56% fertilization), with significant decreases at 4 ppt (73% decrease) and 24 ppt (80.5% decrease). When normalized to 100% for the highest fertilization rate observed in each species (Fig. 3), the fertilization rate for Baltic herring in 8 ppt was significantly higher than that for Pacific herring ($p<0.001$), while the fertilization rate for Pacific herring at 16 ppt and 24 ppt was significantly higher than that for the Baltic herring ($p<0.05$ and $p<0.01$ respectively).

Salinity also affected embryonic development as measured by ability of the embryo to complete development through hatching. As with fertilization, the optimal salinity for hatching in the Pacific herring was 16 ppt with significant decreases observed at 4 and ≥28 ppt (Fig. 4). The optimal salinity for hatching in the Baltic herring was shifted downward to 8 ppt with significant decreases in 4 ppt and >24 ppt, almost identical to that observed for fertilization in this species (Fig. 5). Salinity had no observable effect on events during development in either species; i.e. first cleavage, gastrulation, optic vesicle formation, or initiation of retinal pigmentation. In Pacific herring, scoliosis of larvae was a more frequent finding in embryos incubated at less than optimal salinities, averaging about 10% at 4 and 28 ppt as compared to 1-2% in 16 ppt (Griffin et al. 1998), and none of these larvae survived. In Baltic herring, the fewest scoliotic larvae were observed at 8 ppt (5%), while 9-15% of larvae in 4, 12, 16,and 20 ppt were scoliotic. At salinities ≥ 24 ppt, the incidence of scoliosis increased significantly ($p<0.05$), exhibiting 25% scoliosis at 24 ppt, 34% at 28 ppt, and 62% at 32 ppt.

Cross-fertilization studies between Pacific herring sperm and Baltic herring eggs resulted in an optimal salinity range of 8-20 ppt, with a maximum fertilization rate of 50% in 8 ppt seawater (Fig 6A). Fertilization was significantly decreased ($p<0.05$) at 4 ppt, and in ≥28ppt. The salinity tolerance range for development was much narrower than that for fertilization, with an optimal salinity of 8-12 ppt of hatching, and significantly lower ($p<0.05$) hatching success in 4 ppt, and ≥16 ppt (Fig. 6B).

Effects of varying ion concentrations on fertilization in Pacific herring

Embryos incubated in varying concentrations of Na^+ exhibited a dose response effect with inhibition of fertilization at $[Na^+]_o \leq 55$ mM and ≥ 440 mM ($p<0.05$) (Fig. 7A). Fertilization was unaffected in $[K^+]_o$ of 1.25-10 mM, but was significantly inhibited when K^+ was omitted from the medium, exhibiting a decrease of 94.1% as compared to 5 mM K^+ (concentration in ½ ASW) (Fig. 7B). Similarly, varying the concentration of Ca^{2+} did not have a significant effect on fertilization unless completely omitted from the medium; in ½ CaF, the fertilization rate was 4% versus 89 % for ½ ASW (containing 5 mM Ca^{2+}), an inhibition of 97% (Fig. 7C). Finally, deletion of Mg^{2+} from the medium also significantly inhibited fertilization ($p<0.05$) by 31% as compared to 25 mM Mg^{2+} (concentration in ½ ASW), (Fig. 7D).

Na^+ and K^+ had a synergistic effect on fertilization. In the presence of 5 mM K^+ (concentration in ½ ASW), no significant effect on fertilization was observed in $[Na^+]_o$ of 220-385 mM ($p<0.05$) (Fig. 8). Increasing the concentration of K^+ to 10 mM (concentration in full-strength ASW) resulted in a significant reduction in fertilization for $[Na^+]_o \geq 330$ mM.

Effect of salinity on Pacific herring sperm motility

Reduced and elevated salinities were found inhibit SMIF induced motility initiation. The optimal salinity for initiation was 16-24 ppt, similar to that for fertilization. A significant decrease in motility initiation was observed at salinities ≤ 12 ppt and ≥ 28 ppt.

Field exposures

Not unexpectedly, salinity measurements for both the Fort Baker and Paradise Cove field sites were highest at the beginning of the winter season, generally becoming lower as the rainy season progressed, and salinities at Paradise Cove (low salinity site) were generally lower as compared to the Fort Baker yacht harbor (data not shown). Temperature, which ranged from 7.5-14°C was not significantly different between the two sites. The effects of salinity on hatching in outplanted embryos generally correlated with those obtained in laboratory exposures. Hatching success was optimal at salinities of 16-20 ppt, with decreasing rates at salinities <10 ppt and > 25 ppt (Fig. 9). Analysis of cation concentrations by the UC Veterinary Diagnostic Lab indicate that the molar concentration of cations at the varying salinities remained constant with respect to the others, with a ratio of 44 Na^{+}: 1 K^{+}: 1 Ca^{2+}: 5 Mg^{2+}. The concentration of each cation was also similar to concentrations used to make up ½ ASW, the optimal media for fertilization and development in the Pacific herring.

Discussion:

Pacific herring are known to spawn over a broad range of salinities, ranging from 5-35 ppt (Blaxter and Holliday, 1963; Haegele and Schweigert, 1985). However, selection of spawning sites characterized by differing salinity profiles may be a function of the salinity exposure history of specific reproductive stocks. Although some populations spawn in lowered salinities, other stocks spawn in salinities approaching those of full-strength seawater (Dushkina, 1973; Kijima *et al.*, 1992; Kobayashi,. 1993). Selection of sites with differing salinity profiles has been shown to occur not only in populations that

are widely separated from each other, but in populations that are not geographically separated (Kobayashi, 1993). For example, a distinct population of herring in Japanese waters has been found to spawn in lowered salinity estuaries, while another population has been found to spawn in oceanic waters (Kijima *et al.*, 1992; Kobayashi, 1993), and in British Columbia, some herring stocks spawn in reduced salinities in the Strait of Georgia, while others spawn along the west coast of Vancouver Island which is thought to be exposed to oceanic salinities (Hay *et al.*, 1984, Haegele and Schweigert, 1985, Hay, 1990). Within the Strait of Georgia, distinct spawning stocks have also been identified which exhibit fidelity to specific spawning sites, even though the salinities of these sites do not differ markedly (Hay *et al.*, 1984; Hay, 1985). Within this region, segregation of spawning stocks is thought to occur when herring move onto the spawning grounds and hold for a period of time (Hay, 1985). Freshwater input during the winter and spring months, which coincides with spawning in this species, influences both spawning behavior and reproductive success in at least some herring reproductive stocks (Taylor, 1971; Barnhart, 1988). In addition, there is evidence that low salinity seawater can stimulate spawning in the field (Rabin and Barnhart, 1986) as well as in the laboratory (Outram, 1951).

In this study we show that the range of salinity tolerances for fertilization and embryonic development in Pacific herring from San Francisco Bay is 12-24 ppt, with an optimal salinity for both events of ~16 ppt. These results were observed both in the laboratory (Griffin et al. 1998), as well as in the field. This narrow range of salinities is similar to British Columbia herring, in which the salinity tolerance range is reported to be 12-26 ppt (Alderdice and Velsen, 1971; Alderdice and Hourston, 1985). A broader range

of salinities (10-36 ppt) has been described for spawning Pacific herring in the White Sea (Dushkina (1973). This may reflect the presence of several distinct populations, each with a narrow salinity tolerance, or reflect the capacity of the larger population to adjust to varying salinities. The narrow tolerance range observed in Pacific herring from San Francisco Bay is supported by data collected by the California Department of Fish and Game during their annual spawning surveys. In the late 1980's and early 1990's, herring spawning biomass declined dramatically, coincident with an increase in salinity in the bay due to diversion of freshwater and several years of drought (Spratt *et al.*, 1992; Trujillo et al. 1992). With the return of more normal rainfall years, spawning biomass increased substantially. Thus it would seem that salinity is at least one of the factors governing the spawning behavior of Pacific herring in San Francisco Bay.

Population differences in salinity tolerances have also been reported for the Atlantic herring, *C. harengus*, which in general exhibit a broader fertilization and developmental salinity tolerance range than does the Pacific herring. (Blaxter and Holliday, 1963; Ojaveer, 1981; Haegele and Schweigert, 1985). The Baltic herring, *C. harengus membras*, is a subspecies of the Atlantic herring, yet displays reproductive strategies that resemble those of the Pacific herring. Unlike Atlantic herring sperm, which are motile at spawning (Evans and Geffen, 1998), Baltic sperm are similar to Pacific sperm in that they are not motile at spawning, but initiate motility upon contact with an egg-derived protein isolated from Baltic and Pacific herring chorions. This protein co-migrates with SMIF (molecular weight 105 kDa), the egg-derived protein previously found to initiate motility in Pacific herring sperm (Pillai *et al.*, 1993; Griffin *et al.*, 1996), and is recognized by anti-SMIF antibodies. As with Pacific herring, Baltic

herring sperm also initiate motility in low sodium seawater (1/4 NaF). The salinity tolerance range for Baltic herring is lower than that for Pacific herring, with an optimal salinity for fertilization and development of 8 ppt as compared to 16 ppt for Pacific herring. Since adult Baltic herring reside in lowered-salinity seawaters (4-10 ppt) throughout the year (Haapala and Alenius, 1994), this suggests that the optimal salinity for fertilization and development in the Baltic herring reflects the salinity exposure of the adult herring prior to spawning. A similar exposure regimen may be implicated for Pacific herring that spawn in lowered-salinity seawaters. While adult Pacific herring reside in oceanic waters for most of the year, the tendency of these herring to migrate to and hold in lowered salinity seawaters prior to spawning may reflect a requirement for adjustment to these lower salinity seawaters.

Cross-fertilization of Baltic eggs with Pacific sperm resulted in a fertilization rate of 50-52% in 8-20 ppt seawater and a hatching success of 55-60% in 8-12 ppt salinities. While the optimal salinity ranges for fertilization and hatching were somewhat broader than for Baltic or Pacific herring alone, similar reductions in fertilization and hatching were observed at reduced (4 ppt) or elevated (>24% for fertilization, >16% for hatching) salinities. Cross-fertilization of Pacific eggs with Baltic sperm has previously been described (Rosenthal *et al.*, 1978). In their study, the maximal fertilization rate was 21% and maximal hatching rate of 12.5%, however, fertilization was only assayed at 20 ppt, not over a broad range of salinities.

The inhibition of fertilization observed in this study at salinities <8 and >24 ppt most likely reflects the decrease in SMIF-induced sperm motility initiation observed at these same salinities, and correlates with previous observations that fewer motile sperm

are observed in the micropyle region of eggs at higher and lower salinities (Griffin *et al.*, 1998). It has previously been shown that Pacific herring sperm maintain the ability to fertilize longer in lowered salinity seawater than in full-strength seawater (Yanagimachi and Kanoh, 1953; Morisawa *et al.*, 1992). While the sperm of most teleosts becomes motile upon contact with the external media, motility is of limited duration due to hypo-osmotic swelling or hyper-osmotic shrinking of the sperm (Morisawa, 1994). Immotility of Pacific herring sperm until contact with the egg occurs may represent a strategy to ensure that sperm are not activated until eggs are present in the vicinity, but still remain capable of fertilizing an egg once activated.

Hatching success of embryos outplanted at the two field sites paralleled those observed in laboratory exposures (Griffin *et al.*, 1998), with an optimal salinity for development of 16 ppt. With both decreasing (4-8 ppt) and increasing salinities (28-32), hatching success was decreased. Salinity measurements were not monitored continuously, therefore it is impossible to predict the daily salinity regime that outplanted embryos were exposed to. Clearly, continuous monitoring of salinity and temperatures at the field sites would have provided more accurate information on temporal and spatial variation in salinity exposure, particularly with regard to tidal cycle and during storm periods.

In addition to effects on fertilization and hatching success, salinity may have more subtle effects on development. Previous studies have shown that salinity may delay time to hatch (Griffin et al. 1998). As in this study, embryos exposed to 3.5 and 28 ppt seawaters had lower hatching rates than embryos incubated in 14 ppt seawater. While hatching peaked at 10 days for embryos in 14 ppt, embryos at 28 ppt continued to hatch

through day 11. Such delays in hatching could result in lower survivability if yolk sac reserves are reduced below the level needed to survive hatching and the early larval period prior to onset of feeding. As in this study Griffin *et al.*, (1998) also documented a higher incidence of scoliotic larvae at reduced and elevated salinities, as well as a high incidence of embryos that initiated hatching, but died prior to completion of this process. In addition, abnormal larval behavior (lack of vigorous swimming) was observed at reduced and elevated salinities. These morphological and behavioral abnormalities would certainly result in decreased survivability of herring larvae, impacting ability of larvae to avoid predators or to actively pursue and capture prey.

This study has demonstrated that salinity plays a major role in early reproductive events in Pacific herring from San Francisco Bay. Natural factors that influence the salinity profile of the bay, such as drought or El Nino conditions, also impact herring reproductive behavior as well as early reproductive events in this ecologically and economically important species. In addition, diversion of water for agricultural and urban uses can also impact the reproductive success of this species. While natural events (drought, El Nino events) are not subject to intervention, water policy issues should be addressed to ensure that Pacific herring, as well as other species dependent on estuaries and bays for habitat and reproduction, are protected.

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Fig. 1: SDS-PAGE and immunoblotting of Pacific and Baltic SMIF. Lane A: Coomassie blue stained gel of Pacific SMIF exhibiting a band at 105 kDa. Lane B: the corresponding immunoblot labeled with anti-SMIF (Pacific) antibody. Lane C: Coomassie blue stained gel of Baltic SMIF with a band at 105 kDa. Lane D: the corresponding immunoblot showing recognition of the Baltic 105 kDa band by anti-SMIF (Pacific) antibody

Fig. 1

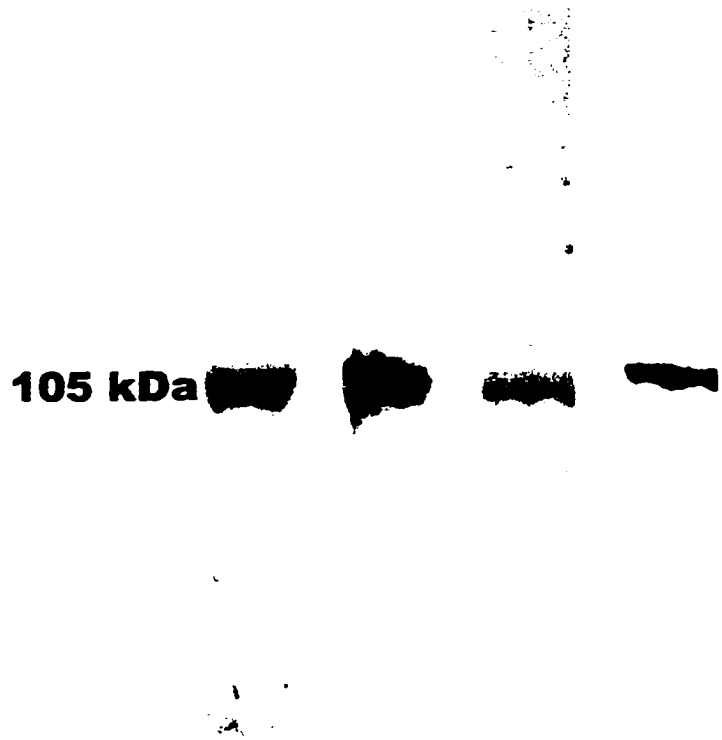


Fig. 2: Effect of salinity on fertilization in Pacific herring from San Francisco Bay, CA ■ and Baltic herring from Airisto Sound, Finland ●. The optimal salinity for fertilization in the Baltic herring was 8-16 ppt, as compared to 12-24 ppt in the Pacific herring. Fertilization was significantly inhibited at 8 and $\geq$28 ppt in Pacific herring and at 4 and > 24 ppt in Baltic herring ($p<0.05$). (a = significance between fertilization rates in Pacific herring; b = significance between fertilization rates in Baltic herring).

Fig. 2

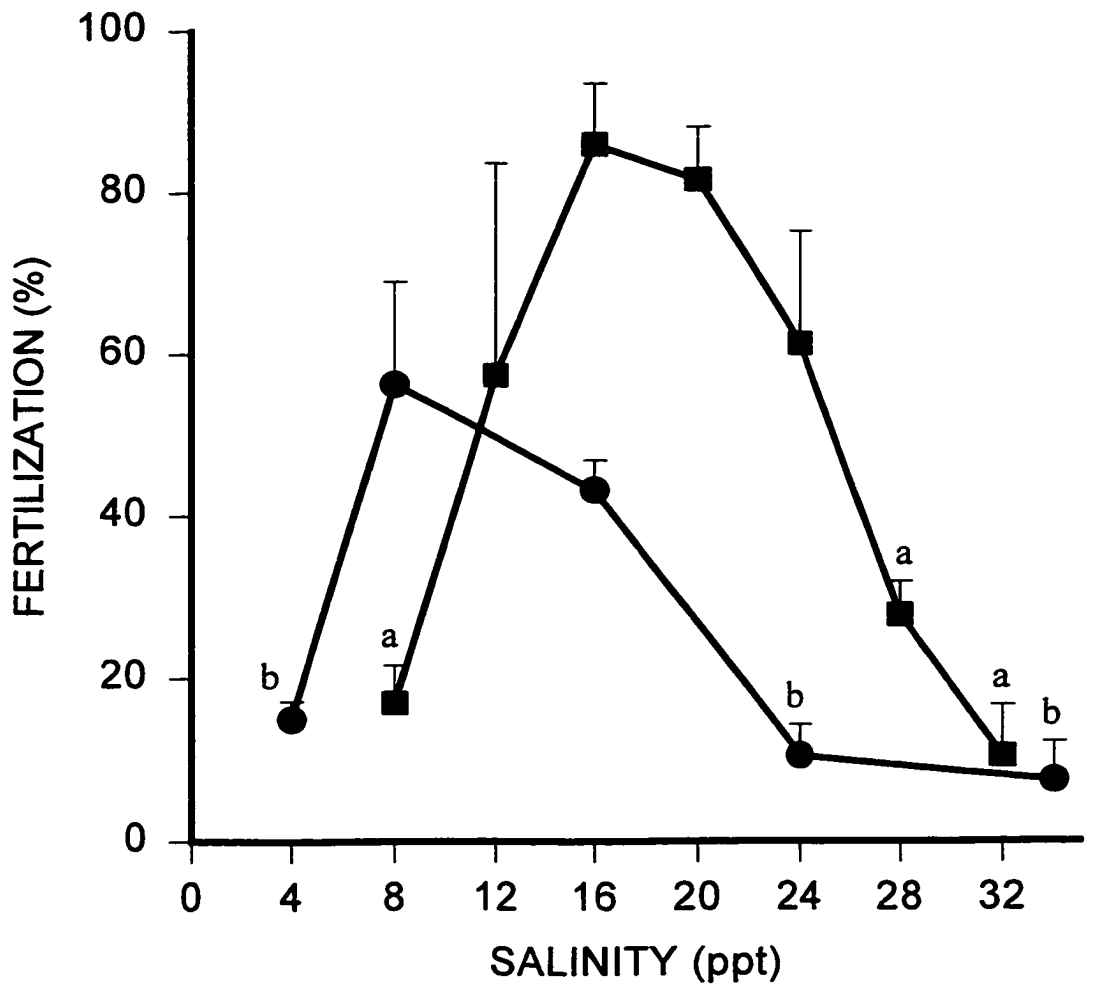


Fig. 3: Normalized fertilization percentages for Pacific herring ■ and Baltic herring ● at different salinities. The salinity tolerance curve for Baltic herring is shifted downward by 8 ppt from that for Pacific herring. Letters indicate significance between fertilization success at selected salinities. a-a $P < 0.05$ at 16 ppt. b-b = $P < 0.01$ at 24 ppt. c-c = $p < 0.001$ at 8 ppt.

Fig. 3

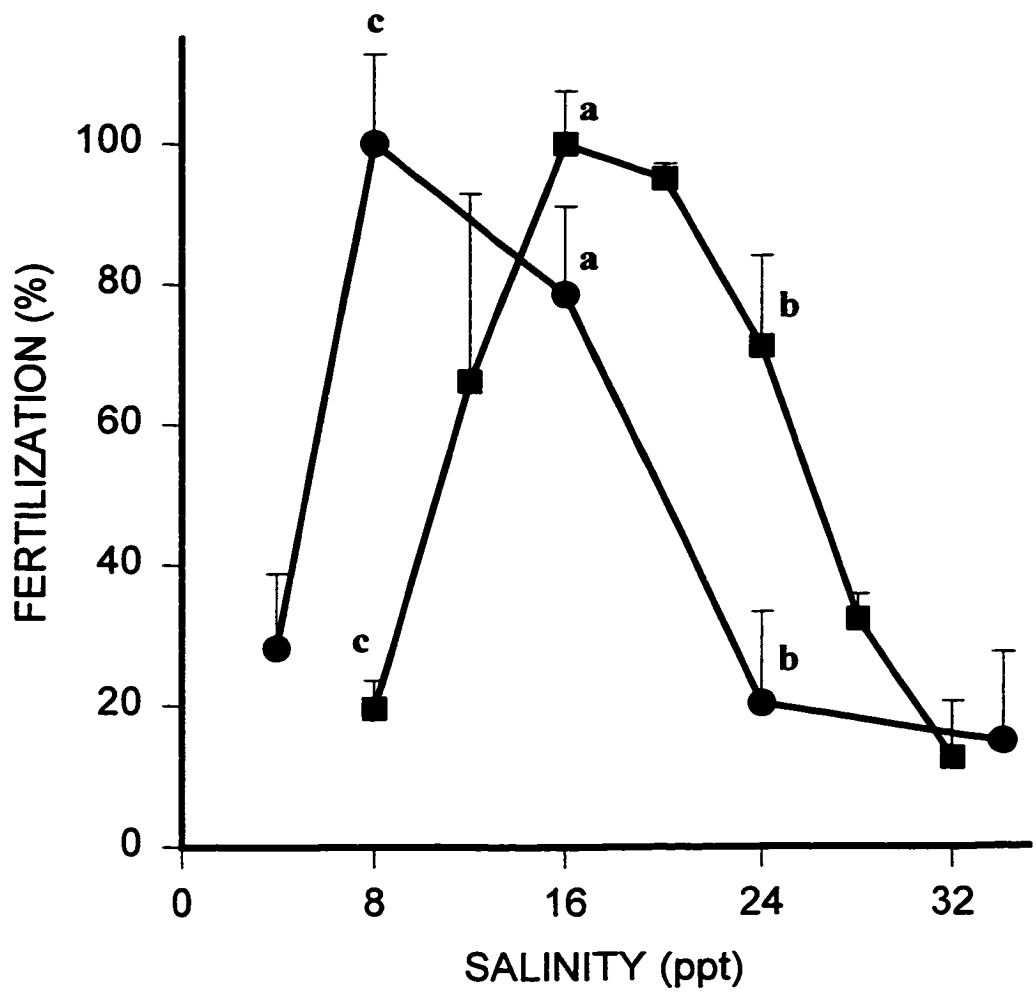


Fig. 4: Effect of salinity on hatching of Pacific herring. The optimal salinity for hatching was 12-20 ppt with significant decreases in hatching success at 4, 28, and 32 ppt Asterisks indicate significance at $p < 0.01$.

Fig. 4

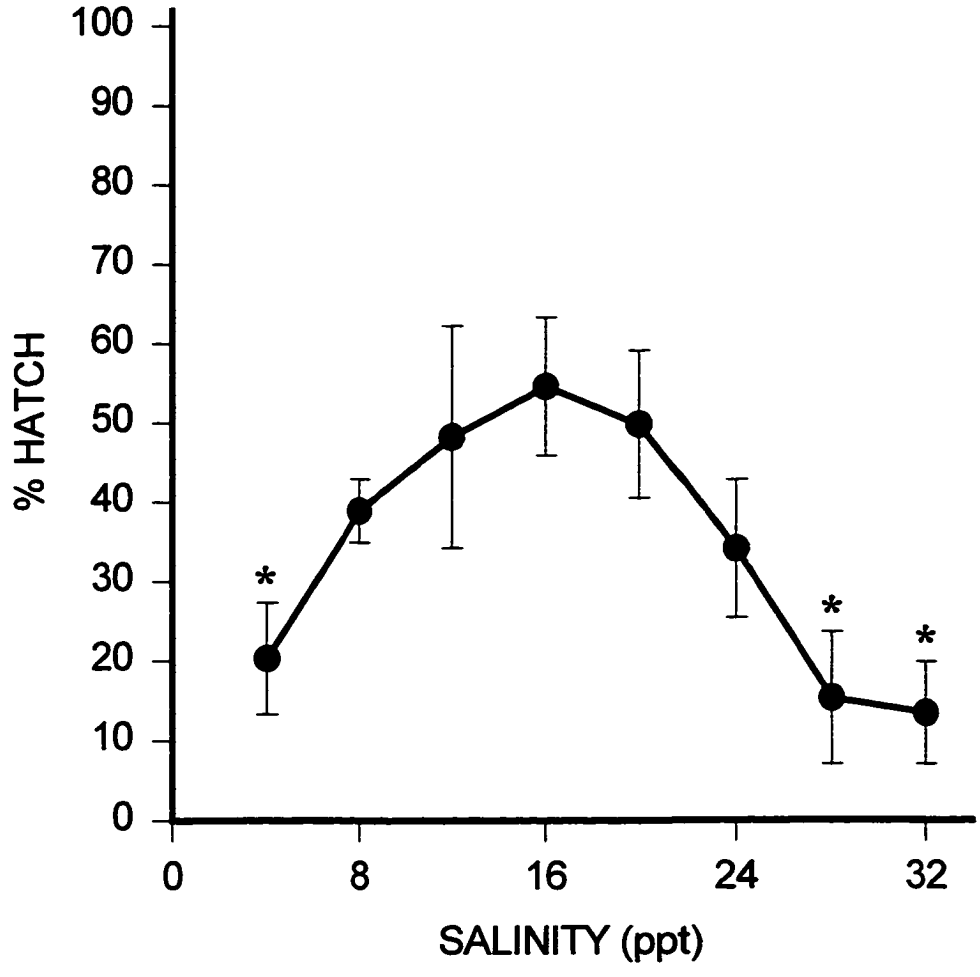


Fig. 5: Effect of salinity on hatching in Baltic herring. The optimal salinity for hatching was 8 ppt, with significant decreases in hatching at $\geq$24 ppt. Asterisks indicate significance at $p<0.05$.

Fig. 5

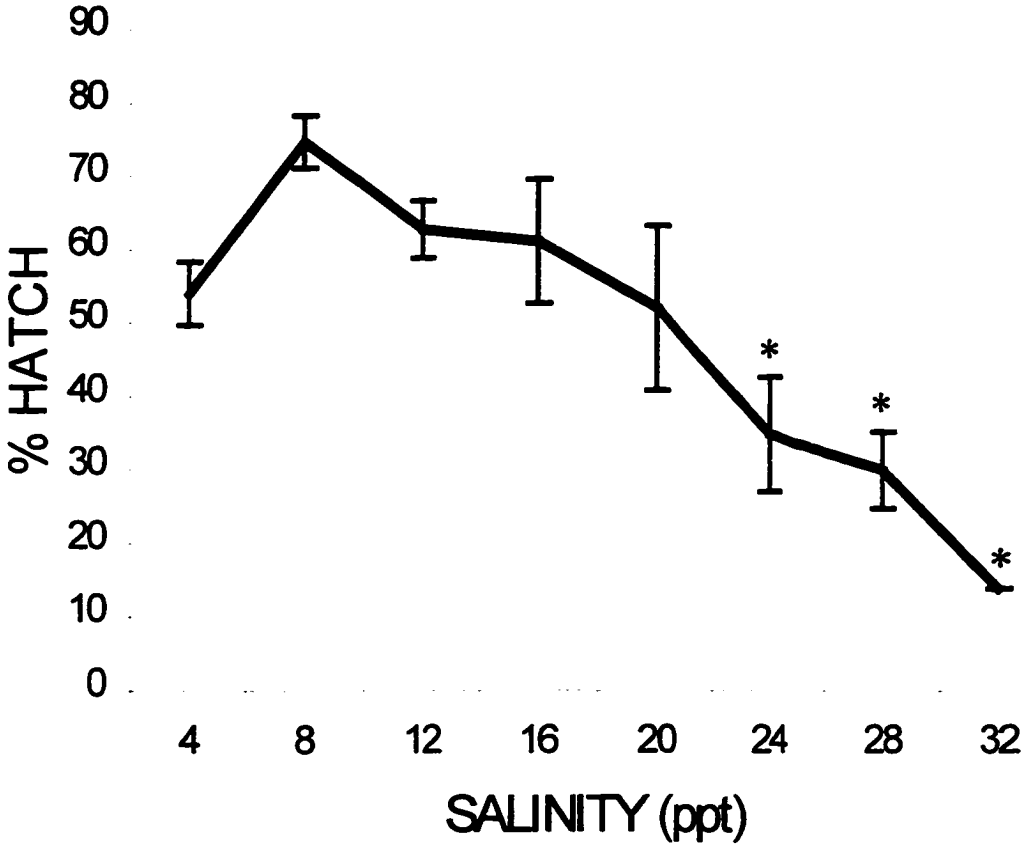


Fig. 6: Effect of salinity on fertilization and hatching in Baltic herring eggs fertilized with Pacific herring sperm. 6A. The optimal salinity for fertilization was 8-20 ppt with a significant decrease in fertilization observed at 4 ppt and $\geq$ 28 ppt. 6B: The optimal salinity for hatching was 8-12 ppt, with significant decreases in hatching success at 4 ppt and $\geq$16 ppt. Asterisks indicate significance at $p<0.05$.

Fig. 6A

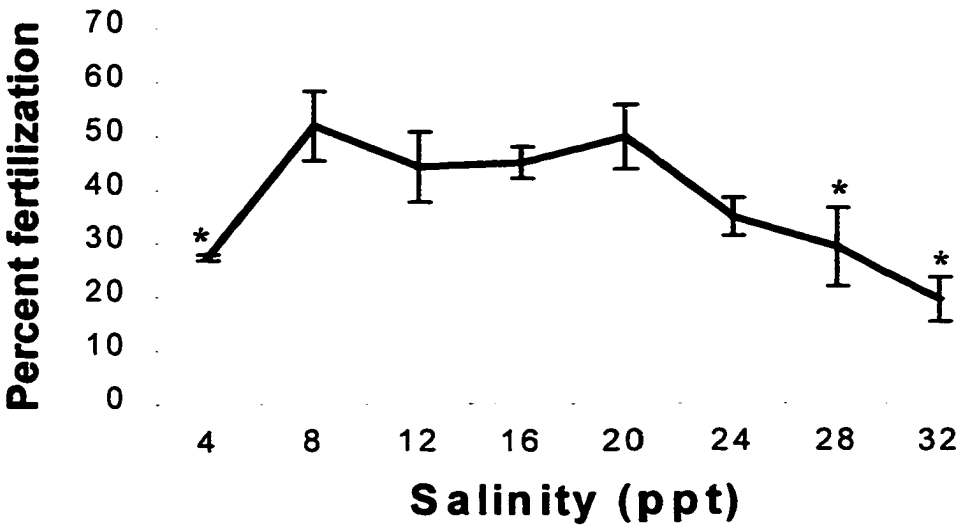


Fig. 6B

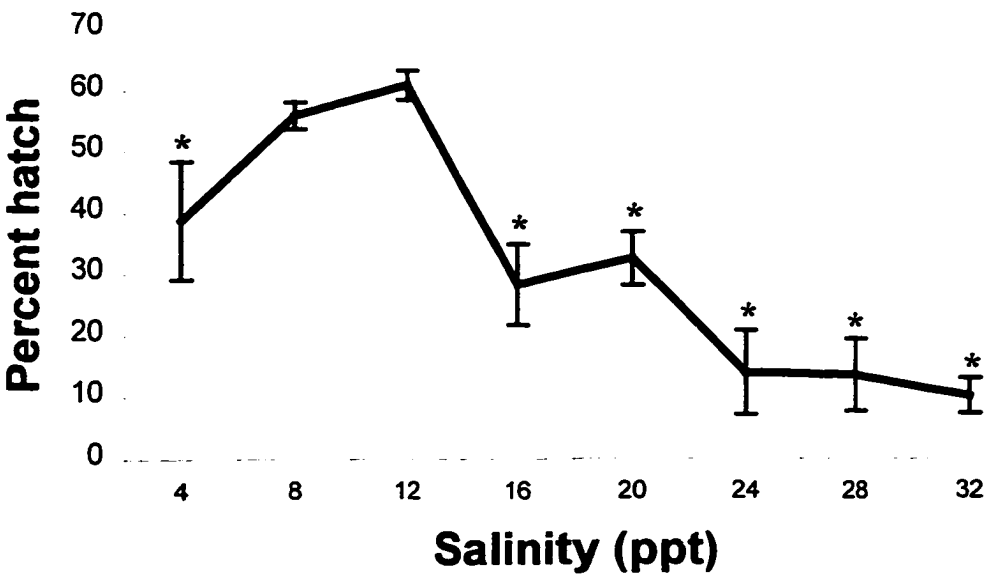


Fig. 7: Effect of cations on fertilization. Eggs and sperm were incubated in varying concentrations of Na^+, K^+, Ca^{2+}, or Mg^{2+} and fertilization assessed. 7A. Effect of Na^+. Fertilization was significantly inhibited in Na^+ concentrations $\leq$55 and $\geq$ 440 mM. 7B. Effect of K^+. Fertilization was significantly decreased only in KF medium. 7C: Effect of Ca^{2+}. Fertilization was significantly decreased only in ½ CaF medium. 7D. Effect of Mg^{2+}. Fertilization was slightly decreased in ½ MgF. (asterisks indicate significant difference at $p<0.05$)

Fig. 7A

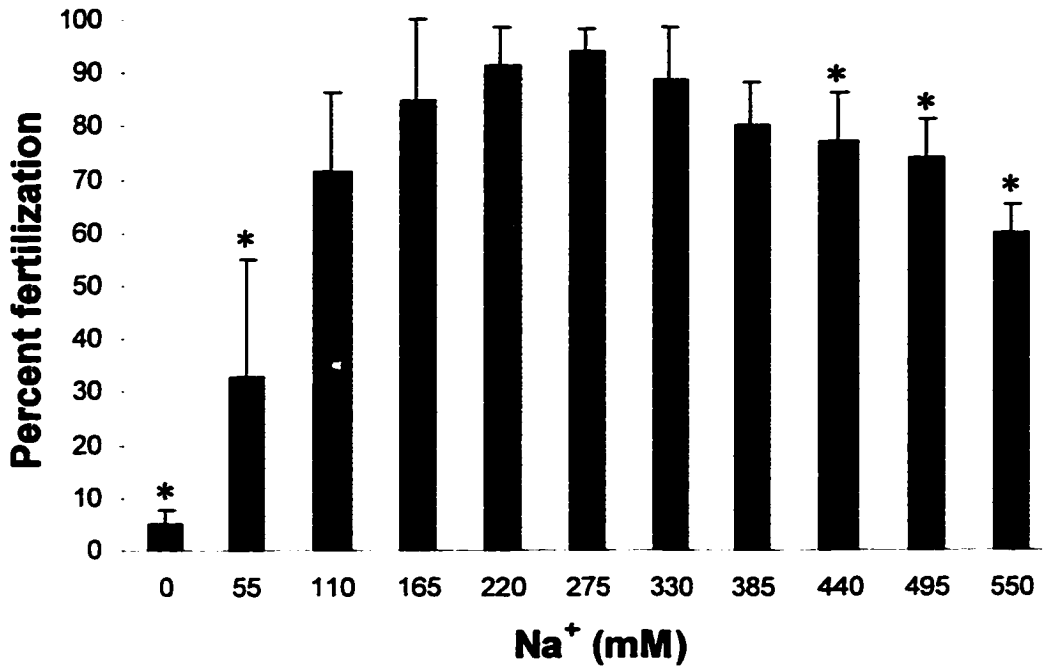


Fig. 7B

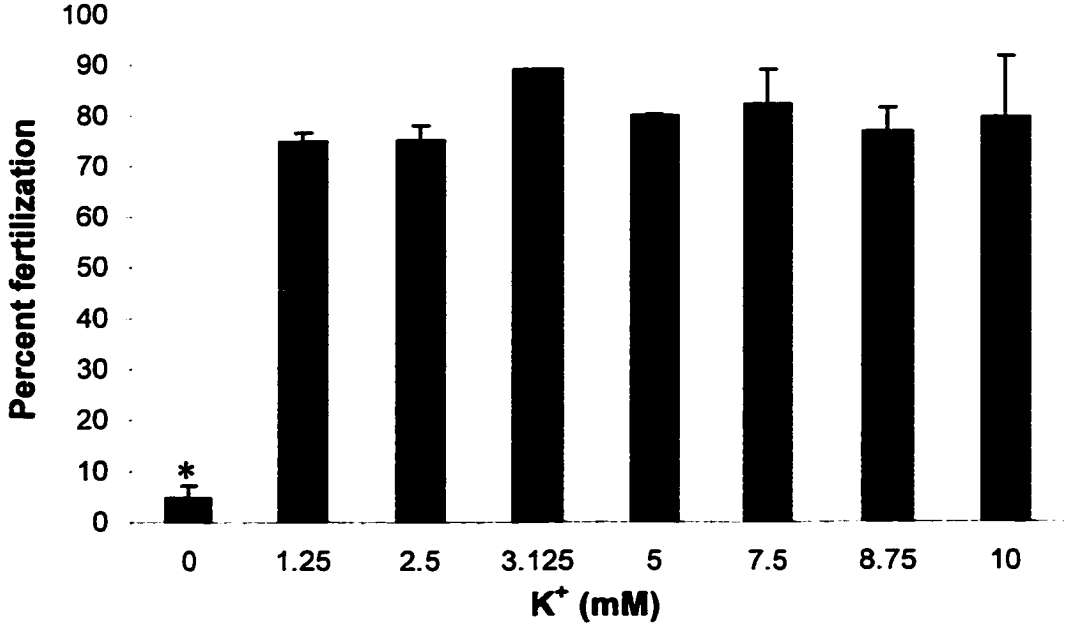


Fig. 7C

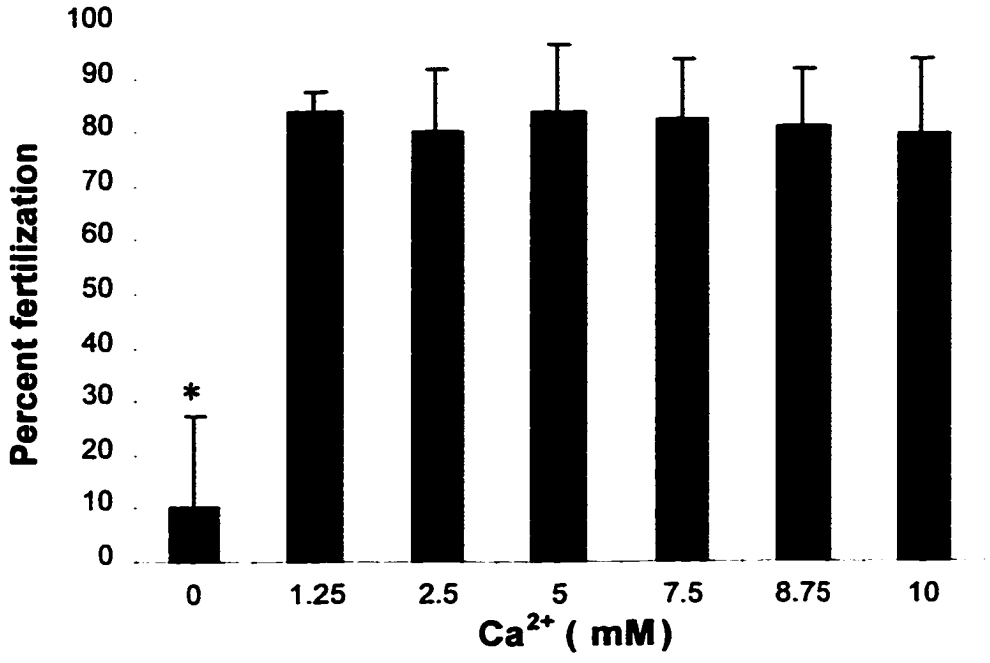


Fig. 7D

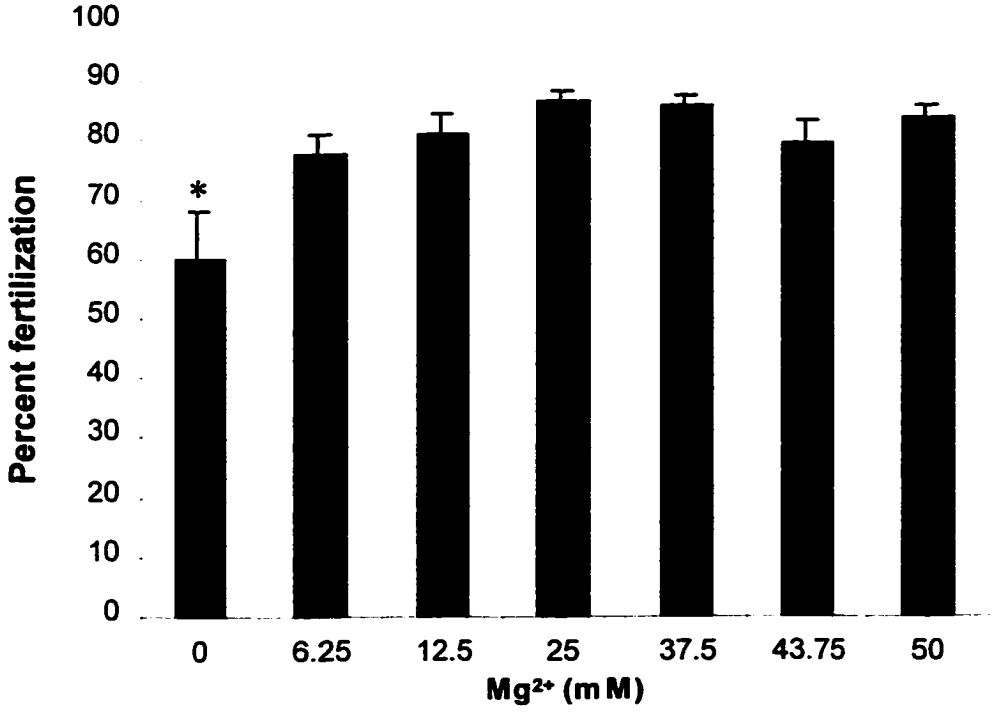


Fig. 8: Synergistic effect of Na^+ and K^+ on fertilization. Sperm and eggs were incubated in ½ ASW containing 5 or 10 mM K^+ and increasing concentrations of Na^+ and fertilization assessed. Fertilization was significantly affected in [Na^+] of 440-550 mM when the [K^+] was 5 mM (concentration in ½ ASW). When the [K^+] was increased to 10 mM, fertilization was significantly inhibited in [Na^+] of 330-550 mM. (a indicates significance at $p < 0.05$ for 5 mM K^+; b indicates significance at $p < 0.05$ for 10 mM K^+).

Fig. 8

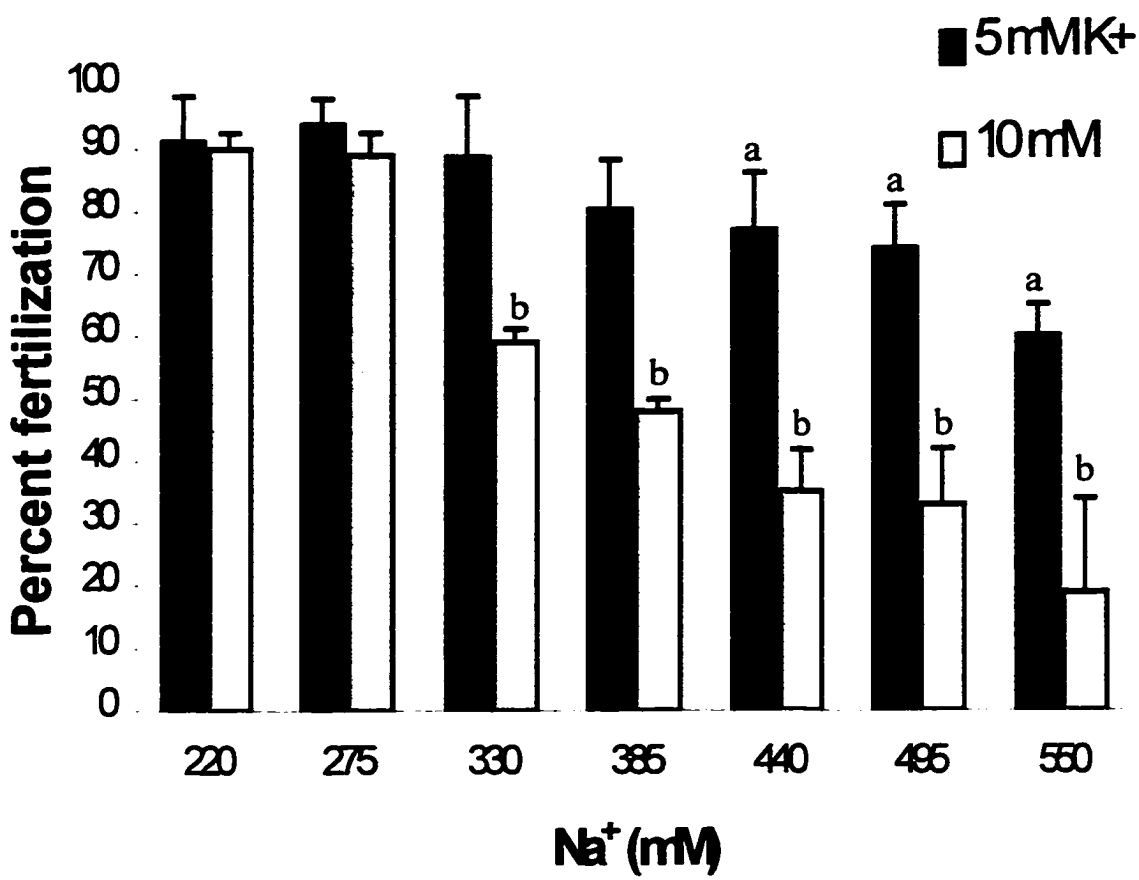


Fig. 9: Effect of salinity on SMIF-induced sperm motility initiation. Sperm motility initiation was inhibited by salinities $\leq$12 ppt and $\geq$28 ppt, with optimal initiation at 16-24 ppt. Motility was scored as 1 = <25% motility; 2 = 25-50% motility; 3 = 50-75% motility; and 4 = >75% motility.

Fig. 9

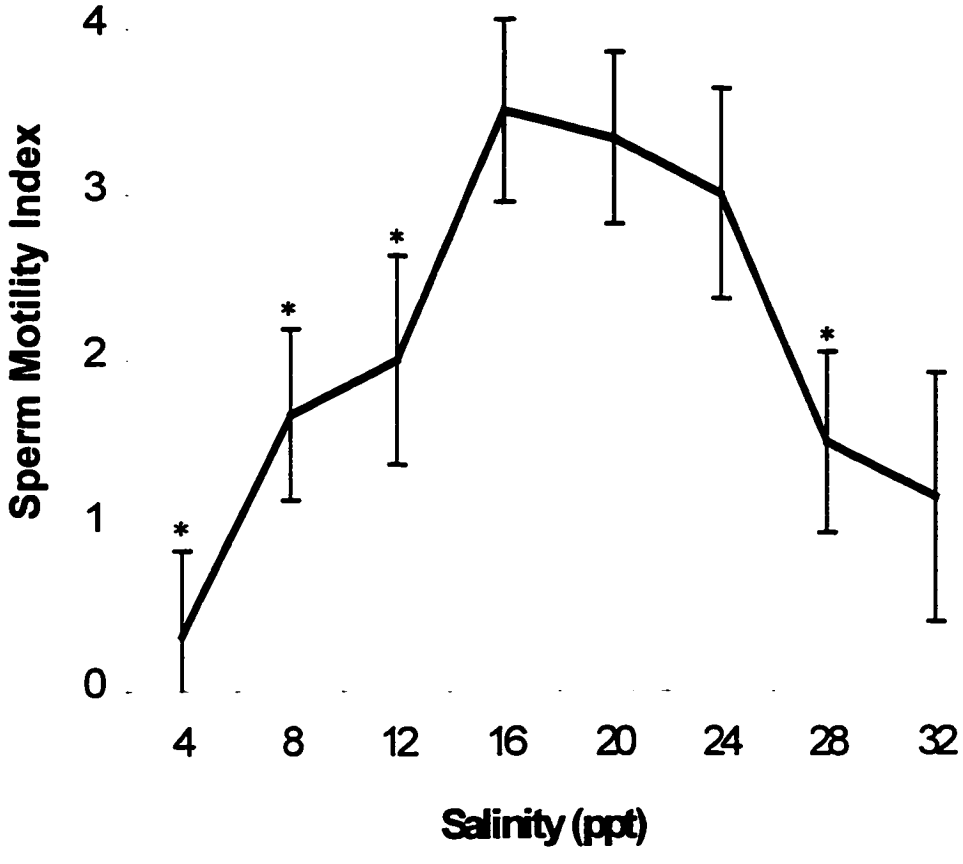
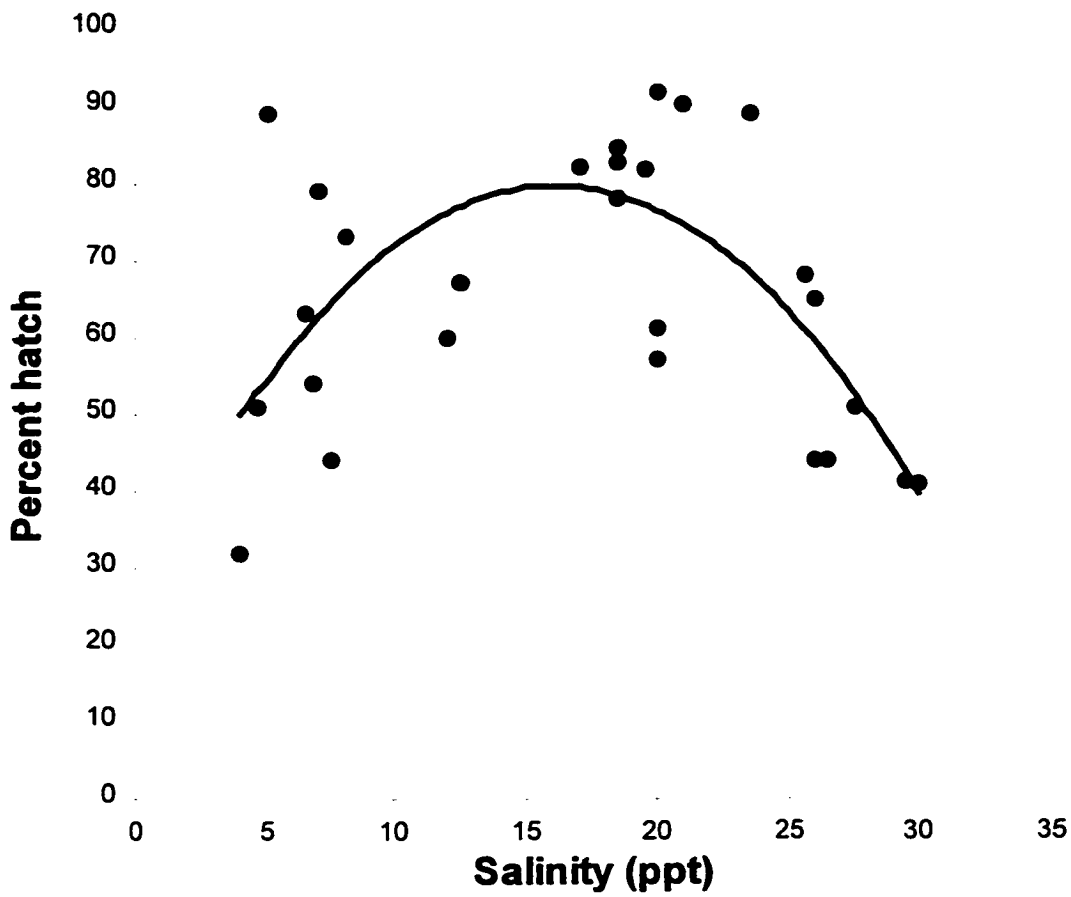


Fig 10: Hatching success of embryos deployed at two field sites in San Francisco Bay, CA in relation to salinity profiles. The optimal salinity for hatching is 16-20 ppt. Salinities <10 ppt or >25 ppt generally resulted in lower hatching rates.

Fig. 10



CHAPTER 2

A Novel Physiological Adaptation in Sperm from the Pacific Herring: Reverse Na^{+}-Ca^{2+} Exchange Regulates Motility Initiation by an Egg-Derived Molecule

ABSTRACT

Unlike most animals, sperm of the Pacific herring (*Clupea pallasi*) are immotile at spawning, and motility is only initiated after contact with a sperm motility initiating factor (SMIF), an egg-derived protein localized to the micropyle region of the herring egg. Motility is also initiated in 1/2 low sodium ("sodium-free") seawater (1/2 NaF). The mechanisms by which SMIF initiates sperm motility, as well as the effects of intracellular and extracellular ion manipulation on motility initiation were investigated. Motility initiation and increases in intracellular calcium $[Ca^{2+}]_i$ were induced by the addition of SMIF, and both were inhibited by the Ca^{2+} channel blockers nifedipine and flunarazine, as well as the sodium-calcium (Na-Ca) exchange inhibitor, bepridil. SMIF also induced a decrease in intracellular sodium ($[Na^+]_i$), and a plasma membrane depolarization in control 1/2 filtered sea water (FSW). The depolarization was completely inhibited in full-strength FSW, or 1/2 FSW containing elevated Na^+. The depolarization was also inhibited by bepridil and slightly by nifedipine. The resting membrane potential was found to be dependent primarily on extracellular Na^+ ($[Na^+]_o$), with some contribution by extracellular K^+ ($[K^+]_o$). In the absence of SMIF, motility could be initiated in 1/2 NaF, but only in the presence of at least 1 mM $[Ca^{2+}]_o$. In addition, an increase in $[Ca^{2+}]_i$ was observed only upon addition of Ca^{2+} to sperm diluted into 1/2 low sodium-calcium free seawater (1/2 NaCaF). Dilution into 1/2 NaF with Ca^{2+} resulted in a gradual increase in $[Ca^{2+}]_i$ as compared to the immediate increase induced by SMIF, while sperm diluted into 1/2 FSW exhibited no increase in $[Ca^{2+}]_i$. SMIF induced an efflux of Na^+ in motile sperm. In the absence of SMIF, Na^+ efflux was observed upon dilution of sperm to1/2 NaCaF only upon subsequent addition of Ca^{2+}, and

this efflux was completely inhibited by bepridil, and partially by flunarazine. Finally, an activator of protein kinase C (PKC), phorbol dibutyrate (PDBu), resulted in an increase in $[Ca^{2+}]_i$ in sperm suspended in 1/2 FSW. This increase was dependent on $[Ca^{2+}]_o$, and could be inhibited by bepridil, flunarazine, and calphostin C.

These results suggest that SMIF-induced motility initiation in herring sperm involves: 1) membrane depolarization; 2) Na^+ efflux and Ca^{2+} influx mediated by a Na-Ca exchange mechanism; 3) the opening of voltage-gated Ca^{2+} channels; and 4) PKC as part of the SMIF-induced activation pathway. This novel mechanism of motility initiation by an egg-derived molecule appears to be an adaptive strategy for maintaining sperm in an immotile state at spawning in a teleost that reproduces in an estuarine environment.

INTRODUCTION

In broadcast spawning animals, synchronous release of gametes typically occurs such that proper mixing of sperm and eggs takes place. The sperm of most external spawners are quiescent within the testes, but initiate motility upon contact with the external medium; the mechanisms by which motility is initiated upon dilution into the external environment vary. Lack of motility within the testes is presumably to conserve limited metabolic resources until such time as they are needed (reviewed by Morisawa, 1994). In marine invertebrates (e.g. sea urchins), dilution into seawater removes seminal fluid CO_2, resulting in an increase in the extracellular pH (Lee *et al.*, 1983). This increase in extracellular pH results in an intracellular H^+ efflux which increases intracellular pH and activates dynein ATPase and subsequent motility (reviewed by Morisawa, 1994). In freshwater teleosts, such as salmonids, sperm motility is initiated by dilution into freshwater which results in decreased extracellular K^+, resulting in: 1) an efflux of intracellular K^+, 2) membrane hyperpolarization, and 3) and an influx of Ca^{2+} (Boitano and Omoto, 1991; Tanamoto, *et al.*, 1994). In many freshwater teleosts (e.g. carp, zebrafish), marine teleosts, amphibians, and some invertebrates, the change in osmolality between the seminal fluid, and either a freshwater (hypo-osmotic) or seawater (hyper-osmotic) environment is the stimulus for motility initiation (reviewed by Morisawa, 1994). This change in osmolality is believed to be related to increases in intracellular Ca^{2+} and pH (puffer fish and flounder), and to changes in intracellular K^+ (puffer fish and zebra fish) (Oda and Morisawa, 1993; Takai and Morisawa, 1995.) .

Unlike the sperm of most organisms, Pacific herring sperm are immotile at the time of spawning in salinities ranging from 8-35 ppt , but initiate motility during sperm-

egg interaction (Yanagimachi and Kanoh, 1953; Yanagimachi, 1957,a,b; Yanagimachi *et al.*, 1992; Oda *et al.*, 1995; Griffin *et al.*, 1996, 1998). These immotile sperm may remain in the water column for many hours prior to sperm-egg interaction and still retain their activation potential (Yanagimachi , 1957). To our knowledge, this feature is unique among most animals possessing motile sperm, although this phenomenon has been reported in two marine invertebrates, the horseshoe crab (Clapper and Brown, 1980; Clapper and Epel, 1982) and the ascidian, *Ciona* (Yoshida *et al.*, 1993). Herring sperm initiate motility upon contact with a region of the chorion immediately surrounding the micropyle due to the presence of a 105 kDa, basic, non-diffusible glycoprotein termed sperm motility initiation factor (SMIF; Pillai *et al.*, 1993; Griffin *et al.*, 1996). In addition to SMIF, low molecular weight diffusible peptides (termed herring sperm activating peptides HSAPs) have also been found to be capable of initiating motility in herring sperm (Morisawa *et al.*, 1992; Oda *et al.*, 1995).

SMIF induced motility is optimal at lowered seawater salinities of 16-24 ppt and is completely inhibited in FSW (~ 32 ppt) (Griffin *et al.*, 1998). This dependency of motility initiation on lowered salinity correlates with the salinity requirements for optimal fertilization and reflects the fact that Pacific herring are marine fish which exhibit adaptations for estuarine spawning. Both $[Na^+]_o$ and $[Ca^{2+}]_o$ have been shown to play a role in either fertilization or sperm motility in herring (Yanagimachi, 1957; Yanagimachi *et al.*, 1992; Griffin *et al.*, 1998). Motility can be artificially initiated in the absence of SMIF using low Na^+ medium (Yanagimachi *et al.*, 1992), while elevated $[Na^+]_o$ inhibits fertilization (Griffin *et al.*, 1998). Sperm motility *in vivo* has been shown to be inhibited in the absence of $[Ca^{2+}]_o$, as was fertilization (Yanagimachi, 1957; Yanagimachi *et al.*,

1992). Based on: 1) artificial motility initiation in 1/2 NaF, 2) inhibition of motility at high $[Na^+]_o$ concentrations, and 3) a requirement for $[Ca^{2+}]_o$ in sperm motility *in vivo*, we investigated whether coupled Na^+ -Ca^{2+} fluxes occur at motility initiation in herring sperm. This study presents the first evidence of a reverse Na-Ca exchange mechanism involved in motility for any spermatozoa. This mechanism represents a specialized adaptation in an estuarine spawning fish whose sperm are immotile at spawning, regardless of the salinity, and only initiate motility at the time of their interaction with an egg-derived molecule(s).

METHODS

Collection of animals and gametes:

Pacific herring were collected by otter trawl from San Francisco Bay and transported to the Bodega Marine Laboratory within 4 hours of collection. Testis were dissected as previously described (Yanigimachi *et al.*, 1992) and stored at 4°C under moist conditions. Sperm for motility experiments and ion measurements were suspended in Herring Ringers consisting of 206 mM NaCl, 7.2 mM KCl, 2.1 mM $CaCl_2$, 3.1 mM $MgCl_2 \cdot 6H_2O$, pH adjusted with 1M $NaHCO_3$ (Yanagimachi 1957) or calcium free Herring Ringers (CaF HR) containing 5 mM EGTA.

Materials

Fluo-3 acetomethoxyester (AM), 3,3'-dipropylthiocarbocyanine iodide (DiS-C_3-(5), sodium green cell permeant (NaGp) and impermeant (NaGi), sodium binding fluorescent indicator-AM (SBFI), potassium binding fluorescent indicator-AM (PBFI) and 20% pluronic F-127 in dimethyl sulfoxide (DMSO) were obtained from Molecular

Probes (Eugene, OR). Nifedipine was obtained from Alamone Labs (Jerusalem, Israel). Monensin, gramicidin, valinomycin, calphostin C and phorbol 12,13-dibutyrate (PDBu) were obtained from Calbiochem (La Jolla, CA). Bepridil, flunarazine, carbonyl cyanide m-chlorophenylhydrazone (CCCP), DMSO, and all other dry chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

One half filtered seawater (1/2 FSW) was prepared by filtering seawater through a 0.45 mm Nalgene filter (Fisher Sci. Pittsburgh, PA) and diluting with ddH_2O. Artificial seawaters were prepared according to Cavanaugh (1975), substituting choline chloride for Na^+ or K^+ in low Na^+ and K^+ free seawaters, and adding 5 mM EDTA or EGTA for Ca^{2+} and Mg^{2+} free seawaters. All seawaters were stored at 13°C. KCl, $CaCl_2$, and NaCl were prepared as 5 M stock solutions in dH_2O.

DiS-C_3(5) (100 mM stock solution) was prepared in DMSO. A stock solution of 1 mM NaGi was prepared in dH_2O. All other fluorescent probes, Fluo-3, NaGp, SBFI, and PBFI, were prepared as 0.5 mM stock solutions in DMSO plus 20% pluronic F-127 in DMSO to facilitate loading.

Stock solutions (10 mM) of nifedipine, flunarazine, bepridil, calphostin C, PDBu, valinomycin and gramicidin were prepared in DMSO. CCCP was prepared as a stock solution of 20 mM in DMSO. Monensin was prepared as a 10 mM stock solution in methanol.

Isolation of SMIF:

Herring chorions were isolated according to Griffin et al. (1996). Herring eggs were suspended in1/2 calcium/magnesium free artificial seawater (1/2 CaMgF) with 0.25% polyvinyl alcohol (PVA) at 4°C and washed 3-5 times to remove ovarian fluid.

Eggs were disrupted 2-3 times in1/2 CaMgF, and the isolated chorions were washed extensively in1/2 CaMgF. Isolated chorions were either used immediately or lyophilized and stored at -70°C for further use. SMIF was isolated as previously described (Pillai *et al.*, 1993). Briefly, isolated chorions were suspended in1/2 ASW (pH 3.5) at 4°C for 30 minutes, with periodic homogenization. The resulting homogenate was adjusted to pH 7.8 and centrifuged at 12,000 g for 15 min. The supernatant was concentrated using 10 kDa MW Centricon 10 microconcentrators (Amicon Co., Beverly, MA). The retentate was aliquoted and used immediately or stored at -70°C for further use. Prior to use of SMIF in experiments, the highest dilution which yielded >75% motility (4+ motility score; see below) was determined and used for all subsequent experiments.

Assessment of Sperm Motility:

Sperm motility was assessed as previously described (Griffin *et al.*, 1996). Twenty µl of each test solution were placed into individual wells of multi-well immunoslides (Polysciences, Inc., PA) and 1 µl of sperm (10^6-10^7 sperm/ml) suspended in HR was added to each well. Motility was observed using darkfield microscopy and a 20x objective lens and evaluated using the following qualitative index: 0 = no motility, 1 = up to 25% motility, 2 = 25-50% motility, 3 = 50- 75% motility, and 4 = >75% motility. For various experiments using activators or inhibitors, sperm were pre-incubated in HR containing the chemical, followed by dilution into the test solution containing the chemical. Sperm motility patterns in response to SMIF, ½ NaF, and ½ FSW were recorded using an MTI Sage CCD camera interfaced to a Macintosh computer and images were captured using NIH Image 1.59 at 16 frames/sec.

Measurement of Membrane Potential:

Membrane potential was measured using the slow cyanine dye, DiS-$C_3(5)$, which accumulates in cells that are hyperpolarized. DiS-$C_3(5)$ exhibits decreased fluorescence emission in hyperpolarized cell membranes, and an increase in emission in depolarized membranes (reviewed by Plasek and Sigler, 1996). To reduce the contribution of mitochondrial membrane potential to DiS-$C_3(5)$ emission spectra, the mitochondrial uncoupling agent CCCP was added. DiS-$C_3(5)$ response was calibrated using varying concentrations of K^+ in 1/2 KF or Na^+ in 1/2 NaF. To maintain osmolality, choline chloride was used to replace K^+ or Na^+ in the respective media. Sperm (10^6/ml) were suspended in the various media, followed by the addition of 0.5 μM DiS-$C_3(5)$ in DMSO and 0.5 μM CCCP. After baseline stabilization, the K^+ ionophore, valinomycin (1μM final), or the Na^+ ionophore monensin (10 μM final) were added to the suspensions based on the methods described in Plasek and Sigler (1996) . DiS-$C_3(5)$ fluorescence was monitored using a spectrofluorometer (Photon Technology Inc.), at 620 nm excitation and 670 nm emission (slit width 5 nm) at 13°C.

To measure the effect of SMIF on membrane potential, sperm (10^6/ml) were suspended in1/2 FSW, 1/2 FSW containing 440 mM Na^+, 1/2 FSW containing 520 mM Na^+, or full strength FSW. To all treatments, DiS-$C_3(5)$ (0.5 μm final) was added, followed by the addition of CCCP (0.5 μM final). After baseline stabilization (~60 sec.), SMIF was added to the sperm suspensions. The Ca^{2+} channel inhibitor nifedipine (50 μM) , or the Na-Ca exchange inhibitor bepridil (50 μM) were added to sperm suspensions (5 min. pre-incubation) prior to DiS-$C_3(5)$, CCCP, and SMIF addition in

experiments which used these inhibitors. Controls consisted of sperm suspensions to which 1/2 FSW was added with DMSO (0.1%) (inhibitor solvent).

Measurement of Intracellular Calcium $[Ca^{2+}]_i$

To measure the effect of SMIF on $[Ca^{2+}]_i$, sperm (10^7/ml) in HR were loaded with 5 μM Fluo-3 for 1 hour at 13°C. Sperm were centrifuged at 900 g (8 minutes at 13°C), one time each over HR containing 10% ficoll, and HR, and re-suspended in fresh HR. Ca^{2+} measurements were made on a spectrofluorometer at 506 nm excitation and 526 nm emission (slit width 5 nm), in 1/2 FSW, 1/2 CaF, 1/2 NaF, or1/2 NaCaF. After stabilization of baseline fluorescence, SMIF or a comparable volume of 1/2 FSW was added. The Ca^{2+} channel blockers flunarazine (10 μM) and nifedipine (100 μM) were used to investigate the type of Ca^{2+} channels involved. $[Ca^{2+}]_i$ concentrations were calculated according to Grynkiewicz *et al.*, (1985) using the equation $[Ca^{2+}]_i = (F-F_{min})/(F_{max}-F)Kd$, where R_{max} = maximum fluorescent response using 50 μM digitonin (20 mM in DMSO stock), R_{min} = minimum fluorescent response using 0.5 M EGTA, pH 8.5 in 1 M Tris-HCl, R = sample response, and Kd = dissociation constant for Fluo-3 (390 nm: Molecular Probes). The K_d for Fluo-3 has not been determined in 1/2 FSW or at 13°C, therefore Ca^{2+} values are presented as percent increase. Sperm motility in all treatments was assessed as described above.

Measurement of Sodium Efflux:

The effect of SMIF on $[Na^+]_i$ was investigated using the cell permeant intracellular Na^+ probe, sodium green (NaGp). Sperm in HR (10^8/ml) were loaded with 5 μM NaGp for 2 hours at 13°C, followed by centrifugation at 900 g in HR (2x) for 8

minutes, and resuspended in fresh HR. Sperm (10^6/ml) were suspended in ½ FSW or ½ FSW containing SMIF and $[Na^+]_i$ was monitored at excitation 507, emission 532. In some experiments, sperm were suspended in ½ FSW, and following baseline stabilization, SMIF or 1/2 FSW was added to the sperm suspensions and the change in fluorescence recorded.

Na^+ efflux was also measured using the impermeant extracellular Na^+ probe, sodium green (NaGi) at excitation 507 nm, and emission 532 nm. Sperm (10^6/ml) were suspended in ½ NaCaF (in which sperm are immotile) followed by the addition of 5 μM NaGi. After baseline stabilization, 20 mM Ca^{2+} (5 mM final) was added to the sperm suspension and the change in fluorescence recorded. A comparable volume of 1/2 NaCaF was added to the control. To measure the effect of Ca^{2+} channel or Na-Ca exchange inhibitors on the Ca^{2+} induced Na^+ efflux, sperm were incubated with 20 μM flunarazine, 20 μM bepridil, or DMSO (solvent control) for 5 minutes prior to measurements. Sperm motility was assessed for these treatments as described above.

Measurement of Intracellular Na^+ and K^+

$[Na^+]_i$ and $[K^+]_i$ were measured using the dual wavelength fluorescent probes SBFI and PBFI. Sperm (10^8/ml) in HR were loaded with 5 μM dye for 1 hour at 13°C, then centrifuged over 10% ficoll in HR (1x) and HR (1x) and resuspended in fresh HR. Sperm were suspended in 1/2 NaKF containing increasing concentrations of Na^+ or K^+, followed by the addition of 10 μM gramicidin to equilibrate $[Na^+]_i$ and $[Na^+]_o$, or $[K^+]_i$ and $[K^+]_o$ based on the methods described in Negulescu and Machen (1990), and fluorescence was monitored on a spectrofluorometer at excitation 340 nm and 380 nm, and emission at 505 nm.

Cell signaling events in herring sperm:

To investigate the role of protein kinase C (PKC) in sperm function, sperm were loaded with the $[Ca^{2+}]_i$ indicator, Fluo-3, as described above. Sperm were suspended in 1/2 FSW or 1/2 CaF, with or without flunarazine (10 μM), bepridil (10 μM) or the PKC inhibitor, calphostin C (50 μM). After baseline stabilization, the PKC activator, PDBu (10 μM), was added, and Fluo-3 fluorescence was measured on a spectrofluorometer. Sperm motility was assessed for the different treatments as described above.

RESULTS

Sperm motility:

In 1/2 NaF without SMIF, increasing concentrations of Na^+ resulted in a decrease in motility, such that at $\geq$150 mM Na^+, motility was completely inhibited (Fig1a), while in the presence of SMIF, Na^+ concentrations $\geq$350 mM were found to significantly inhibit motility (Fig 1b). Unlike 1/2 NaF, 1/2 CaF and 1/2 KF did not initiate motility in the absence of SMIF in this study (Table 1; also reported by Yanagimachi *et al.*, 1992). While motility initiation in1/2 NaF required a minimum of 1 mM Ca^{2+}, increasing the concentration of Ca^{2+} (10 mM in this study; 30 mM in Yanagimachi *et al.*, 1992) above the normal level of 1/2 ASW (5 mM) had no effect on either initiation or inhibition of motility (Table 1). Initiation of motility in ½ NaF was not dependent on the presence of K^+, and was unaffected by the addition of 1-10 mM K^+. Concentrations of 15-20 mM K^+ did inhibit motility. Sperm pre-incubated with the Ca^{2+} channel blockers nifedipine (10 μm), and flunarazine (10 μm) failed to initiate motility in the presence of SMIF.

Membrane Potential

Membrane potential was found to be affected by SMIF and was sensitive to Na^+ and K^+. Addition of SMIF to sperm suspended in 1/2 FSW with DiS-C_3(5) resulted in a depolarization response (increase in fluorescence intensity) as shown in Fig 2. SMIF-induced depolarization was significantly inhibited by 50 μM bepridil (67% inhibition), but only partially inhibited by 50 μM nifedipine (6% inhibition). SMIF-induced depolarization was not observed in sperm suspended in FSW or in ½ FSW containing an additional 300 mM Na^+ (~520 mM total), and was reduced in sperm suspended in 1/2 FSW containing an additional 220 mM Na^+ (~440 mM total) (Fig 3).

Since herring sperm motility is initiated in 1/2 NaF in the absence of SMIF, we investigated the effect of $[Na^+]_o$ on the resting membrane potential. Since K^+ is known to be the major contributor to membrane potential in most cells, we also investigated the role of K^+ in herring sperm resting membrane potential. Calibration of dye response to K^+ using the K^+ ionophore, valinomycin, resulted in hyperpolarization of sperm suspended in 1/2 KF and 1/2 KF containing 25 mM K^+, while 50 mM K^+ resulted in depolarization of the membranes (Fig. 4A). However, with $[K^+]_o$ >100-150 mM, hyperpolarization was again observed (Fig. 4B). A similar effect was observed in response to $[Na^+]_o$. For sperm suspended in 1/2 NaF, a hyperpolarization was observed in $[Na^+]_o$ of 0-35 mM Na^+, with depolarization occurring at Na^+ > 35 mM (Fig. 5A). At Na^+ concentrations >100 mM, hyperpolarization was again observed (Fig. 5C).

Ionic Changes Associated with Motility Initiation

Intracellular Ca^{2+}. As described above, herring sperm motility initiation, both in the presence of SMIF and in 1/2 NaF requires the presence of $[Ca^{2+}]_o$. To determine

whether a Ca^{2+} influx occurs, the Ca^{2+} indicator Fluo-3, was used to measure changes in $[Ca^{2+}]_i$ upon addition of SMIF to sperm suspended in1/2 FSW. SMIF induced a 2-6 fold increase in $[Ca^{2+}]_i$, over sperm suspended in1/2 FSW (Fig 6A). When Ca^{2+} was omitted from the medium, an increase in $[Ca^{2+}]_i$ only occurred when Ca^{2+} (5 mM final) was added back to the medium (Fig. 6B). The SMIF-induced increase in $[Ca^{2+}]_i$ was inhibited by the Ca^{2+} channel blockers 100 μM nifedipine (74.6% inhibition), and 10 μM flunarazine (89.6% inhibition) (Fig. 7). The increase in $[Ca^{2+}]_i$ in response to SMIF or 1/2 NaF differed in magnitude and in velocity. A gradual increase in $[Ca^{2+}]_i$ was observed in sperm suspended in 1/2 NaF (in the absence of SMIF), while an immediate influx was observed in response to SMIF (Fig. 8). The SMIF-induced response was 2-3 times greater than that observed in 1/2 NaF. Differences in sperm trajectories were also observed between SMIF-induced (circular motility pattern) and 1/2 NaF-induced motility (linear motility). In comparison, sperm suspended in ½ FSW do not exhibit an increase in $[Ca^{2+}]_i$ and are not motile.

Intracellular sodium and potassium. $[Na^+]_i$ was measured in sperm loaded with the $[Na^+]_i$ probe, SBFI and suspended in ½ NaKF containing increasing concentrations of Na^+. Addition of 10 μM gramicidin resulted in a decrease in fluorescence intensity in sperm suspended in ½ NaKF and ½ NaKF containing ~10-50 mM Na^+ (data not shown). At concentrations of Na^+ of 50-150 mM, an increase in fluorescence was observed. Since no change in fluorescence is expected at the null point ($[Na^+]_i = [Na^+]_o$), these results suggest that the resting concentration of Na^+ is ~25-50 mM. $[K^+]_i$ was measured using PBFI loaded sperm suspended in ½ NaKF containing increasing concentrations of K^+. Addition of 10 μM gramicidin resulted in a decrease in fluorescence intensity in sperm

suspended in ½ NaKF and ½ NaKF containing 25 mM K^+, and an increase in fluorescence intensity in ½ NaKF containing 50-150 mM K^+, suggesting that the $[K^+]_i$ is 25-50 mM.

Na-Ca Exchange:

Since fluxes in Na^+ and Ca^{2+} were observed during herring sperm motility initiation, and the resting membrane potential was found to be dependent on $[Na^+]_o$, we investigated the possible involvement of a Na-Ca exchange mechanism at motility initiation. The addition of 20 mM Ca^{2+} (5 mM final) to sperm suspended in1/2 NaCaF (in which sperm are immotile) containing the $[Na^+]_o$ probe NaGi resulted in an increase in fluorescence intensity, indicating an efflux of Na^+ (fig 9). This efflux of Na^+ upon addition of Ca^{2+} was completely inhibited by the Na-Ca exchanger blocker, bepridil (20 μM), and partially (65.4% inhibition) by the calcium channel blocker flunarazine (20 μM). Bepridil (50 μM), was also found to inhibit (61.7 % inhibition) SMIF-induced Ca^{2+} influx, as well as motility initiation.

An efflux of Na^+ was also observed in sperm upon exposure to SMIF. In this case, sperm loaded with the cell-permeant $[Na^+]_i$ probe, NaGp, and suspended in 1/2 FSW containing SMIF exhibited a lower fluorescence intensity (indicating a decrease in $[Na^+]_i$) as compared to sperm suspended in ½ FSW (Fig 10A). In separate experiments, addition of SMIF to sperm suspended in ½ FSW resulted in a significantly greater decrease in fluorescence intensity (12%) as compared to addition of ½ FSW to controls which resulted in a 1% decrease in intensity (Fig. 10B).

Intracellular signaling events associated with motility initiation:

In the absence of SMIF, an increase in $[Ca^{2+}]_i$ (125%) was observed upon addition of the PKC activator, PDBu to sperm suspended in 1/2 FSW (Fig. 11). This increase was not observed in the absence of $[Ca^{2+}]_o$. The increase was also inhibited by flunarazine (70.1% decrease) and bepridil (77.4% decrease) (Table 2). In the presence of calphostin C, the PDBu induced influx of Ca^{2+} was inhibited by 76.9%. Calphostin C also inhibited SMIF-induced increase in $[Ca^{2+}]_i$ by 56.7%, as well as sperm motility.

DISCUSSION

In teleost fish sperm, four different triggers for motility initiation have been reported. In salmonids (which spawn in freshwater), sperm motility is inhibited in semen due to high concentrations of K^+ (Morisawa *et al.*, 1983a; Morisawa, 1985) and is triggered by the reduction in extracellular K^+ after dilution into freshwater. In other freshwater-spawning species (including carp, goldfish, crucians, daces, and pejerrey), motility of sperm is triggered by a hypotonic exposure (dilution of semen by freshwater), not specifically as a reduction in any particular ion (Morisawa and Suzuki, 1980; Morisawa *et al.*, 1983b). In a number of marine teleosts, sperm are quiescent in seminal fluid or solutions that are isotonic with seminal fluid and become motile when exposed to a hypertonic solution (e.g. seawater); this includes puffer fish, flounder, Pacific cod (Morisawa and Suzuki, 1980; Oda and Morisawa, 1993), and Atlantic croaker (Detweiler and Thomas, 1998).

The fourth trigger of motility initiation in teleosts has been described only in Pacific herring sperm, which require contact with an egg-chorion ligand(s) (Yanagimachi *et al.*, 1992; Pillai *et al.*, 1993; Morisawa, 1994). Neither hypo- or hyper-osmotic

conditions, nor reduction in extracellular K^+ trigger sperm motility in Pacific herring (Yanagimachi *et al.*, 1992). Pacific herring sperm remain immotile but viable for up to a day after spawning unless contact occurs with one of the egg-associated ligands, SMIF or HSAPs (Yanagimachi and Kanoh, 1953; Yanagimachi, 1957a,b; Yanagimachi *et al.*, 1992; Oda *et al.*, 1995; Griffin *et al.*, 1996, 1998). Sperm motility around the egg chorion as well as *in vitro*, requires a salinity range between 8-24 ppt, that is, the majority of sperm do not become motile as a result of contact with SMIF at salinities outside the 8-24 ppt range (Yanagimachi, 1953; Morisawa *et al.*, 1992; Griffin *et al.*, 1998). This range of permissible salinities correlates with the salinity range for optimal fertilization (Griffin *et al.*, 1998) and reflects the fact that Pacific herring are marine fish which possess adaptations for estuarine spawning. Both extracellular Na^+ and Ca^{2+} are important to fertilization and sperm motility *in vivo* and *in vitro* (Yanagimachi, 1953, 1957; Yanagimachi *et al.*, 1992; Griffin *et al.*, 1998), and results from the present study suggest that at high salinities the $[Na^+]_o$ is too high and at low salinity, the $[Ca^{2+}]_o$ is too low to enable motility initiation.

Membrane Potential Changes

With the exception of Pacific herring, a hyperpolarization of the sperm plasma membrane appears to accompany the initiation of sperm motility in teleosts, whether the trigger is hypo-osmotic change, hyper-osmotic change, or a reduction in the extracellular concentration of a specific ion (e.g. K^+ in salmonids). Detweiler and Thomas (1998) have proposed that an influx of Ca^{2+} as well as a K^+ efflux are important in the activation of sperm motility, and both could be involved in sperm membrane potential changes at activation. In several freshwater fishes, hyperpolarization, caused by a K^+ efflux, is the

first response to a hypo-osmotic change (Tanimoto *et. al.* 1988; Gatti *et al.;* 1990; Boitano and Omoto; 1991; Tanimoto *et al.*, 1994. In salmonids, sperm membrane hyperpolarization is also due to a K^+ efflux (Morisawa, 1985; Boitano and Omoto, 1991; reviewed by Morisawa, 1994; Tanamoto, *et al.*, 1994). Although intracellular Ca^{2+} $[Ca^{2+}]_i$ increases concomitantly with the decrease in external K^+ (Cosson *et al.*, 1989; Boitano and Omoto, 1991), a universal role for external concentrations of this ion has been controversial as there is some evidence of intracellular stores in at least one species (Oda and Morisawa, *et al.*, 1993). Sperm of Pacific herring undergo a membrane depolarization at SMIF-induced activation, rather than a hyperpolarization, and this depolarization is likely due to the efflux of Na^+ and increase in $[Ca^{2+}]_i$.

Membrane potential is dependent on the permeability of plasma membranes to various ions, with K^+ fluxes having a dominant effect in most cells (reviewed by Plasek and Sigler, 1996). The contribution of other ions has been demonstrated in sea urchin sperm, in which membrane potential is sensitive to K^+, Na^+, and Ca^{2+} (Reynaud, *et al.*, 1993) and in mouse sperm, in which K^+ and Ca^{2+} are involved (Espinosa and Darszon, 1995). The membrane potential in herring sperm was found to be dependent on Na^+ and to some extent, K^+. Sperm suspended in 1/2 NaF in the presence of monensin exhibited a hyperpolarization, as would be expected due to efflux of Na^+. Hyperpolarization was also observed for extracellular Na^+ concentrations between 10-35 mM added to 1/2 NaF with monensin,.while at $[Na^+]_o$ concentrations of 45-150 mM, depolarizations were observed (also in the presence of monensin). Concentrations of $Na^+ > 200$ mM resulted in hyperpolarization, probably due to accumulation of positive charge outside of the sperm, resulting in an aggregation of negative charge just inside the plasma membrane to

balance this charge. This hyperpolarization of the membrane may, in part, explain why increasing concentrations of $[Na^+]_o$ or elevated salinities inhibit SMIF induced motility, preventing the SMIF-induced depolarization. Calibration of the DiS-C_3(5) response to Na^+ suggests that the $[Na^+]_i$ may be approximately 35-45 mM Na^+, while measurement of $[Na^+]_i$ using an intracellular Na^+ probe found the concentration to be approximately in the same range at 25-50 mM. This is similar to $[Na^+]_i$ concentrations reported in other teleost sperm (Gatti *et al.*, 1990).

Calibration of the DiS-C_3(5) response (in the presence of valinomycin) to K^+ resulted in hyperpolarization in 0 and 25 mM K^+, a depolarization occurring at 50 mM K^+, and hyperpolarization at $K^+ > 50$ mM; these data suggest that the intracellular K^+ concentration is between 25-50 mM, which is significantly lower than concentrations reported in teleost sperm which are typically 100-150 mM (Gatti *et al.*, 1990). Intracellular K^+ measurements made with a K^+ probe also support this relatively low level of intracellular K^+ in herring sperm. Addition of 5-10 mM K^+ to sperm suspended in 1/2 FSW or 1/2 KF without valinomycin had no significant effect on membrane potential (data not shown). Since > 50 mM K^+ (10 times greater than that found in 1/2 FSW) was required to exhibit any significant effect on membrane potential in the absence of valinomycin, we conclude that K^+ may contribute to the overall membrane potential, but it plays less of a role than Na^+. The lack of a significant response to 5-10 mM K^+ correlates with the observation that 2.5-10 mM K^+ also did not affect sperm motility *in vitro* (this study). However, elevated $[K^+]_o$ (10 mM) may contribute to reduced sperm motility and fertilization when there is elevated $[Na^+]_o$ (>300 mM) (Griffin *et al.*, 1998). In this study, the SMIF-induced membrane depolarization was completely inhibited in

FSW (containing approximately 440 mM Na^+) and partially inhibited in 1/2 FSW (containing approximately 220 mM Na^+) to which an additional 220 mM Na^+ was added. The relative hyperpolarization of the membrane at optimal salinities (220 mM Na^+) may be one mechanism for maintaining the sperm in an immotile state until contact with SMIF is made. Although not involving motility, a similar mechanism has been described in mouse and bovine sperm, where hyperpolarization of uncapacitated sperm may prevent premature acrosome reactions, but allow capacitated sperm to undergo acrosome reactions in response to zona pellucida induced depolarization (Zeng, *et al.*, 1995).

Intracellular Ionic Changes at Motility Initiation

The ionic mechanisms by which motility is initiated in response to both SMIF and1/2 NaF, as well as the maintenance of immotility in sperm diluted in 1/2 FSW, were the focus of this investigation. Both SMIF-induced and 1/2 NaF-induced motility initiation in herring sperm appear to involve an influx of Ca^{2+}, and require at least 1 mM $[Ca^{2+}]_o$. Microscopic observations of sperm motility confirm the requirement for $[Ca^{2+}]_o$, also indicating that a minimum of 1 mM $[Ca^{2+}]_o$ is necessary for motility initiation induction by SMIF or in 1/2 NaF. *In vivo* sperm motility initiation in the herring egg micropylar region has been shown to be dependent on $[Ca^{2+}]_o$ (Yanagimachi and Kanoh, 1953, Yanagimachi *et al.*, 1992). This study has demonstrated that voltage-gated Ca^{2+} channels are present in herring sperm and these appear to be both T-type and L-type in their characteristics based on the use of the well characterized inhibitors flunarazine and nifedipine. In other teleost sperm, an increase in $[Ca^{2+}]_i$ is associated with motility initiation, and in many species an influx of $[Ca^{2+}]_o$ is responsible for the increase at the initiation of motility (Tanimoto and Morisawa, 1988; Oda and Morisawa, 1993). An

elevation of $[Ca^{2+}]_i$ from intracellular stores occurs in the hyperosmolality-dependent initiation of sperm motility in at least one species, the puffer fish (Oda and Morisawa, *et al.*, 1993); however there is no evidence of release of Ca^{2+} from intracellular stores in herring sperm based on the results reported here. In other teleost sperm, Ca^{2+} channels appear to be sensitive to osmotic changes, however, the channels in herring sperm are clearly not.

The difference in the increase in $[Ca^{2+}]_i$ between SMIF and 1/2 NaF can be directly related to sperm trajectories in these two treatments, such that SMIF induces a circular pattern of motility, while ½ NaF induces a linear patter of motility. This is consistent with the relationship between increases in $[Ca^{2+}]_i$ and more asymmetric flagellar bending and circular sperm trajectories reported in other systems (Boitano and Omoto, 1991; Cook *et al.*, 1994).

Reverse Na-Ca Exchange

Since motility can be artificially initiated in the absence of SMIF using 1/2 NaF (Yanagimachi *et al.* 1992; Pillai *et al.*, 1994), and since elevated $[Na^+]_o$ inhibits motility initiation, we investigated the coupled flux of Na^+ and Ca^{2+}. The efflux of Na^+ observed in response to SMIF or in 1/2 NaF in the presence of $[Ca^{2+}]_o$, as well as the inhibition of SMIF-induced motility by elevated $[Na^+]_o$ and by the Na-Ca exchange inhibitor, bepridil, suggest that initiation of motility involves a reverse Na-Ca exchange mechanism. To our knowledge, this study provides the first evidence for such an exchanger in sperm.

A role for a linked Na^+ and Ca^{2+} movement has not been observed in other animal sperm to our knowledge. In fact, Na^+ has only been linked to motility in sea urchin sperm where initiation involves Na^+-H^+ exchange (Lee *et al.*, 1983). We hypothesize that

reverse Na-Ca exchange is involved in SMIF-induced motility initiation in Pacific herring sperm based on: 1) Ca^{2+} influx associated with a Na^{+} efflux; 2) bepridil, a Na-Ca exchange inhibitor inhibits Na^{+} efflux, Ca^{2+} influx, and motility initiation; 3) 1/2 NaF artificially stimulates motility and Ca^{2+} influx; and 4) motility initiation is associated with a membrane depolarization rather than the hyperpolarization observed in other teleost systems, and it is known that depolarization of the membrane is associated with reverse Na-Ca exchange (Blaustein *et al.*, 1991). In addition, in preliminary studies we have observed that Ca^{2+} influx and motility initiation are not affected by ouabain or tetrodotoxin, thus ruling out Na^{+}-K^{+} ATPase and at least some voltage-gated Na^{+} channels as transport mechanisms, at least in initiation of motility (these or others could be involved in maintenance of motility) (data not shown).

Na-Ca exchange mechanisms in a variety of tissue types have been described in vertebrate and invertebrate systems (reviewed by Philipson and Nicoll, 1993). In most of these systems Na-Ca exchange serves to pump Ca^{2+} out of the cell (forward Na-Ca exchange). Reverse Na-Ca exchange has been described in mammalian smooth muscle, in this case with the influx of calcium thought to contribute to increasing muscle tone. A reverse Na-Ca exchange mechanism has also been described in pancreatic B cells (Herchuelz and Plasman, 1991), and barnacle muscle cells (Bittar and Nwoga, 1990; Rasgado-Flores *et al.*, 1991; Blaustein *et al.*, 1991). Depolarization of the sperm membrane in response to SMIF is similar to that seen in barnacle muscle fibers, whereby depolarization of the membrane during activation appears to favor Ca^{2+} influx via Na-Ca exchange (Blaustein *et al.*, 1991). An increase in $[Ca^{2+}]_i$ through voltage-gated Ca^{2+} channels often occurs immediately prior to Na-Ca exchange in squid axons for example,

and is associated with an enhancement of Ca^{2+} influx via the Na-Ca exchanger (DiPolo *et al.*, 1982; DiPolo and Beauge, 1986). As such, while voltage-gated Ca^{2+} channel blockers such as nifedipine do not inhibit the Na-Ca exchanger, they may impact its overall activity by reducing $[Ca^{2+}]_i$ through inhibition of voltage-gated channels (reviewed by Herchuelz and Plasman, 1991). The linkage of an increase in $[Ca^{2+}]_i$ via L- and/or T-type channels, as a result of SMIF-activation, to the activation of the Na-Ca exchanger in herring sperm, may be similar to other non-muscle cells.

The specific sequence of ionic events which occur as a result of SMIF binding to its receptor on the midpiece of the sperm (Griffin *et al.*, 1998) is not clear at present. It is likely that SMIF induces membrane depolarization and an increase in $[Ca^{2+}]_i$ via voltage-gated channels. A depolarization of the sea urchin sperm membrane has been observed in response to egg jelly (Garcia-Soto *et al.*, 1987). This depolarization is thought to trigger the influx of Ca^{2+} required for the acrosome reaction in sea urchin sperm. It is tempting to speculate that the membrane depolarization induced by SMIF may be the triggering event for motility initiation by allowing the influx of Ca^{2+} required for motility, coupled to Na^+ efflux.

Bepridil, an antianginal agent that inhibits Na-Ca exchange (reviewed by Gill *et al.*, 1992) completely inhibited sperm motility and partially inhibited Ca^{2+} influx and membrane depolarization in response to SMIF. Additionally, Ca^{2+} induced efflux of Na^+ was also inhibited by bepridil. Since bepridil can also inhibit Ca^{2+} influx through receptor mediated Ca^{2+} channels, it is tempting to speculate that SMIF may also induce a Ca^{2+} influx by binding to membrane Ca^{2+} channels. The fact that flunarazine, a T-type Ca^{2+} channel blocker also inhibited SMIF-induced increases in $[Ca^{2+}]_i$, but only partially

inhibited Na^+ efflux would seem to suggest that influx of Ca^{2+} through Ca^{2+} channels alone is not responsible for triggering the efflux of Na^+ out of sperm, as has been proposed for mouse sperm (Espinosa and Darszon, 1995) and sea urchin sperm (Lee *et al.*, 1983).

Our results suggest that PKC has a role in Na-Ca exchange and motility initiation in herring sperm. The PKC activator PDBu (10 μM) initiated sperm motility and Ca^{2+} influx (125% increase dependent on $[Ca^{2+}]_o$). Calphostin C, a PKC inhibitor, as well as flunarazine and bepridil, inhibited the PDBu-induced $[Ca^{2+}]_i$ increase. Calphostin C also inhibited the SMIF-induced increase in $[Ca^{2+}]_i$, as well as motility initiation. These experiments suggest that PKC is involved in SMIF-induced motility initiation.
PKC activation provides an important pathway through which many intracellular signaling events take place (Barritt, 1992). It has recently been shown that PKC activation may be involved in the human sperm acrosome reaction (O'Toole *et al.*, 1996) and in regulation of fowl sperm motility (Ashizawa *et al.*, 1994). In human sperm, the inhibition of calcium channels by nifedipine blocks Ca^{2+} influx and diacylglycerol (DAG) production, both of which are endogenous activators of PKC (O'Toole *et al.*, 1996). This in turn inhibits the phosphorylation of intracellular proteins by PKC. The Na-Ca exchanger is known to be stimulated by increases in Ca^{2+} and, in some systems phosphorylation (reviewed by Reeves, 1992). PKC activation in barnacle muscle is associated with further activation of Na-Ca exchange (Bittar and Nwoga, 1990), PKC phosphorylates the Na-Ca exchanger in heart muscle (reviewed by Philipson and Nicoll, 1993), and phorbol ester dibutyrate, PDBu (an activator of PKC), stimulates Na^+ efflux in barnacle muscle fibers only in the presence of extracellular Ca^{2+} (Bittar and Nwoga,

1990). While there is a phosphorylation site on the cytoplasmic domain of the Na-Ca exchanger (serine residues), a truncated exchanger lacking that domain was still able to transport Na^+ and Ca^{2+} and could still be activated by Ca^{2+} (reviewed by Reeves, 1992). Thus phosphorylation can be an activating mechanism for the Na-Ca exchanger, but is clearly not required as compared to ion gradients and in particular, $[Na^+]_o$ and $[Ca^{2+}]_i$ concentrations.

Sperm Motility Initiation and Fertilization in the Environment

The fact that herring sperm remain immotile after release into water, require contact with egg-associated inducers and appear to utilize mechanisms for motility initiation that have not yet been described for other sperm, fits well with the general reproductive biology of the animal. Male herring spawn from several hours to a day before females, releasing large quantities of sperm ("broadcast spawning") at estuarine spawning grounds (Hourston and Haegle, 1980; Stacey and Hourston, 1982). Thus sperm must survive altered osmotic conditions (dilution of seminal plasma by variable salinity estuarine waters) for extended periods of time and still be capable of becoming motile and fertilizing an egg. The requirement for contact with an egg-associated inducer ensures that sperm do not initiate motility prior to the presence of eggs.

As in other teleost fish sperm, motility initiation in herring is dependent on an increase in $[Ca^{2+}]_i$, however the mechanisms by which this increase occurs appears to be quite different in herring, since the Ca^{2+} channels in herring sperm are apparently not responsive to altered osmolality as is observed in other fish sperm.

We propose that initial control over motility in herring sperm is through Na^+ and Ca^{2+} fluxes which occur via a reverse Na-Ca exchange mechanism. Under low salinity

conditions (~5% seawater) where the $[Na^+]_o$ concentration (<25 mM) would be low enough to induce motility, the $[Ca^{2+}]_o$ would be below the minimum of 1 mM required for motility initiation. At salinities where $[Ca^{2+}]_o$ is high enough, $[Na^+]_o$ is too high and thus inhibitory in the absence of egg-derived molecules.

The circular trajectories resulting from SMIF-induced motility is required for maintaining sperm in the micropylar region in order for successful penetration of the micropylar canal and subsequent fusion with the oolemma (Yanagimachi and Kanoh, 1953; Yanagimachi *et al.*, 1992). We propose that SMIF activates the Na-Ca exchanger through flunarazine and nifedipine-sensitive Ca^{2+} channels resulting in an increase in $[Ca^{2+}]_i$ and subsequent activation of PKC. The large increase in $[Ca^{2+}]_i$ (and accompanying membrane depolarization) and PKC phosphorylation of the Na-Ca exchanger would in turn result in a further increase in $[Ca^{2+}]_i$, and motility with circular trajectories. Circular trajectories ensure that sperm continue to contact the micropyle region and maintain motility by coming in contact with SMIF bound to the chorion (Griffin *et al.*, 1996), and as such, the complexity of motility initiation in sperm from this species is likely to be necessary to facilitate successful fertilization.

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Fig. 1: Effect of $[Na^+]_o$ on initiation of sperm motility. Motility was graded as follows: 0 = <25% motility, 1+ = 25-50% motility, 2+ = 50-75% motility, 4+ = >75% motility. Fig. 1A: Motility initiation in ½ NaF in the absence of SMIF is inhibited with increasing $[Na^+]_o$. At > 25 mM $[Na^+]_o$, motility is significantly inhibited ($p<0.05$). Fig 1B: SMIF induced motility is inhibited by increasing $[Na^+]_o$. A significant reduction in motility is observed at $[Na^+]_o$ > 350 mM ($p<0.05$). Note that a higher concentration of Na^+ is required to inhibit SMIF induced motility versus ½ NaF induced motility.

Fig 1A

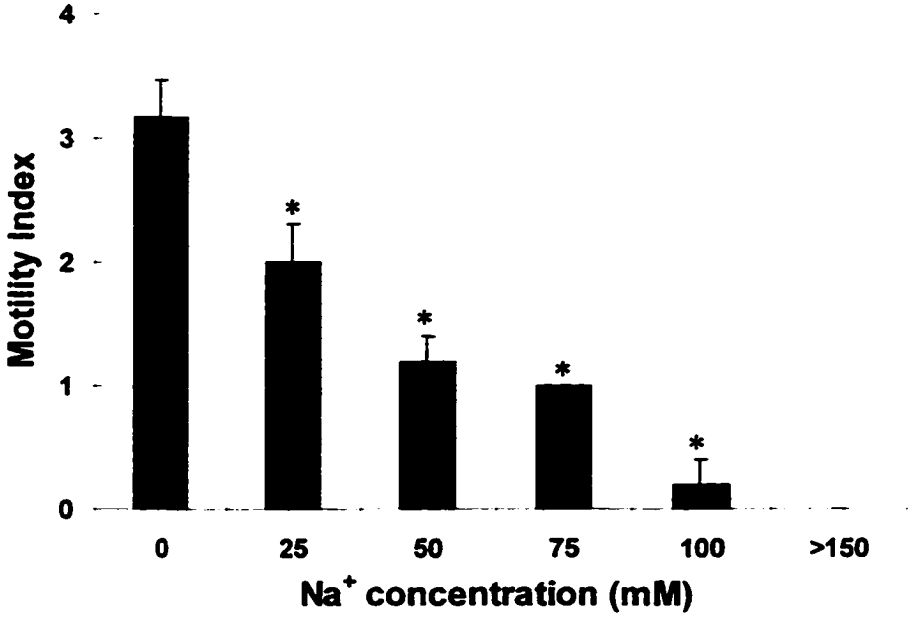

Fig 1B

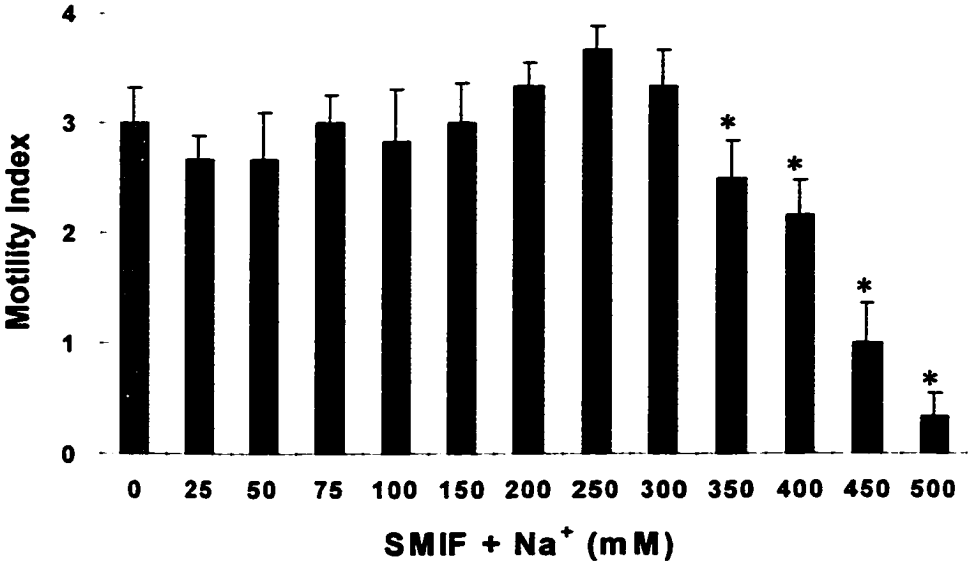

Table 1: Summary of the effects of extracellular ions on sperm motility initiation in the absence of SMIF. Sperm motility is initiated in ½ NaF, but only in the presence of at least 1 mM Ca^{2+}. Increasing concentrations of Ca^{2+} (1-10 mM) or K^{+} (1-10 mM) have no effect on ½ NaF-induced motility. 15-20 mM K^{+} does inhibit ½ NaF-induced motility. Sperm motility is not initiated in ½ CaF, ½ CaF containing 1-10 mM Ca^{2+}, ½ KF, or ½ KF containing 1-20 mM K^{+}. See Fig. 1 for description of motility index.

Table 1

Treatment	Motility Index
1/2 NaF	3-4
1/2 FSW	0
1/2 NaCaF	0
1/2 NaCaF + 0.1 mM Ca^{2+}	0
1/2 NaCaF + 0.5 mM Ca^{2+}	0
1/2 NaCaF + 1-10 mM Ca^{2+}	3-4
1/2 CaF + 1-10 mM Ca^{2+}	0
1/2 NaKF + 0-10 mM K^{+}	3-4
1/2 NaKF + 15-20 mM K^{+}	0-1
1/2 KF + 1-20 mM K^{+}	0

Fig 2: Effect of SMIF on membrane potential. Sperm were suspended in ½ FSW, or ½ FSW containing 50 uM bepridil or nifedipine, followed by the addition of $DiSC_3$-(5) and CCCP (not shown). Upon addition of SMIF (arrow), a depolarization is observed (top tracing), which is inhibited slightly by nifedipine (2^{nd} tracing) and more so by bepridil (3rd tracing). No change in membrane potential is observed in sperm to which ½ FSW is added. (bottom tracing).

Fig. 2

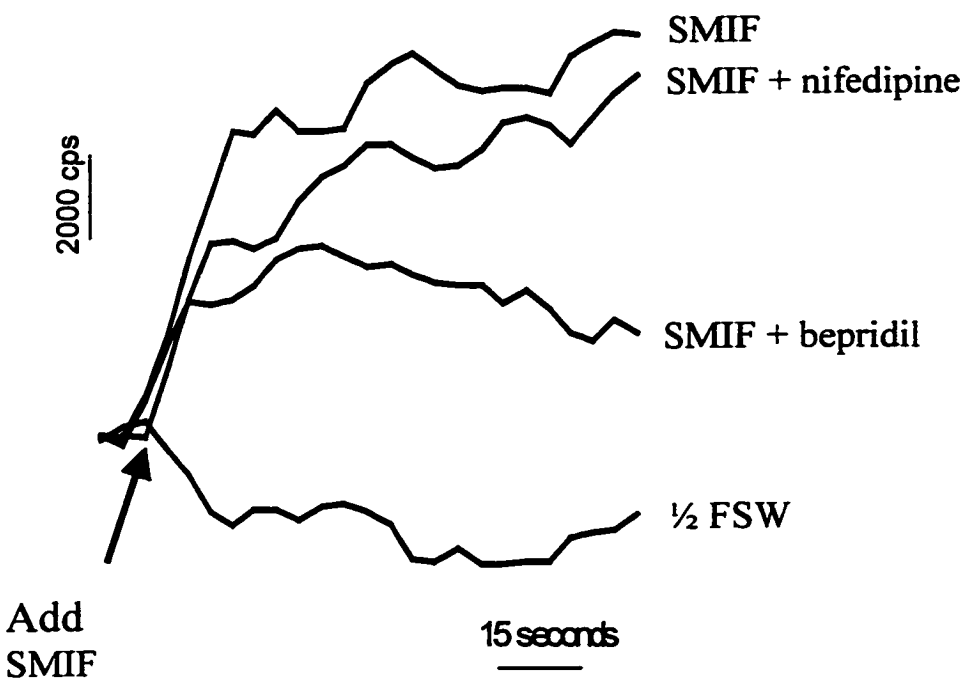

Fig. 3: Effect of salinity on SMIF induced depolarization. Sperm were suspended in ½ FSW, ½ FSW containing 440 mM Na^+ total, ½ FSW containing 520 mM Na^+ total, or FSW, followed by the addition of DiS-C_3(5) and CCCP (not shown). Addition of SMIF (arrow) resulted in a depolarization (upward deflection) in ½ FSW. The SMIF induced depolarization was completely inhibited in FSW and ½ FSW containing 520 mM Na^+ and partially inhibited in ½ FSW containing 440 mM Na^+.

Fig. 3

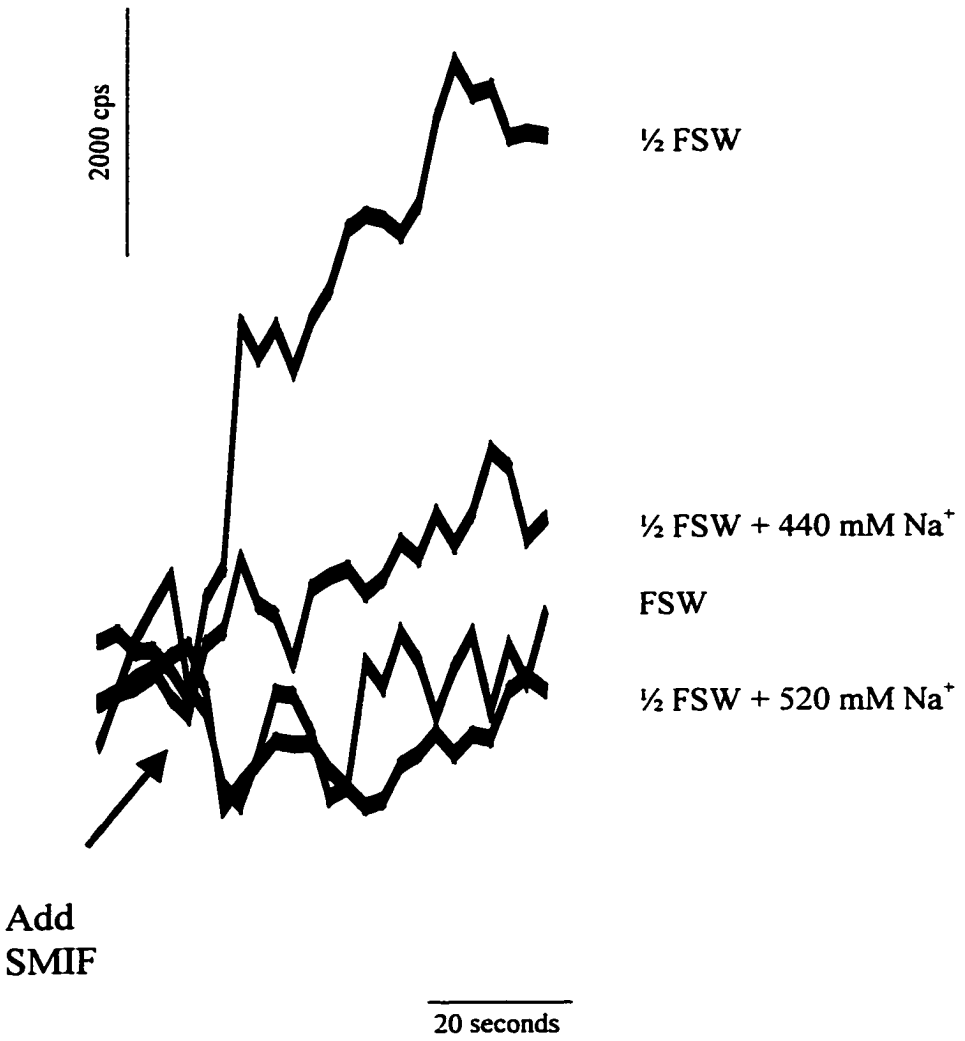

Fig. 4: Effect of K^+ on membrane potential. 4A: Sperm were suspended in varying concentrations of K^+ in ½ KF, followed by the addition of DiS-C_3(5) and CCCP (not shown). Following baseline stabilization, 1 μM valinomycin (arrow) was added. A hyperpolarization (downward deflection) was observed in 0 and 25 mM K^+ and a depolarization (upward deflection) was observed with 50 mM K^+. 4B: Sperm were suspended in ½ KF, followed by the addition of DiS-C_3(5) and CCCP where shown. Following baseline stabilization, 1 μM valinomycin was added, followed by the addition of 50 mM increments of $K^=$ (arrows). 50 mM K^+ resulted in a depolarization, while additional 50 mM increments (100-150 mM) resulted in hyperpolarization.

Fig. 4A

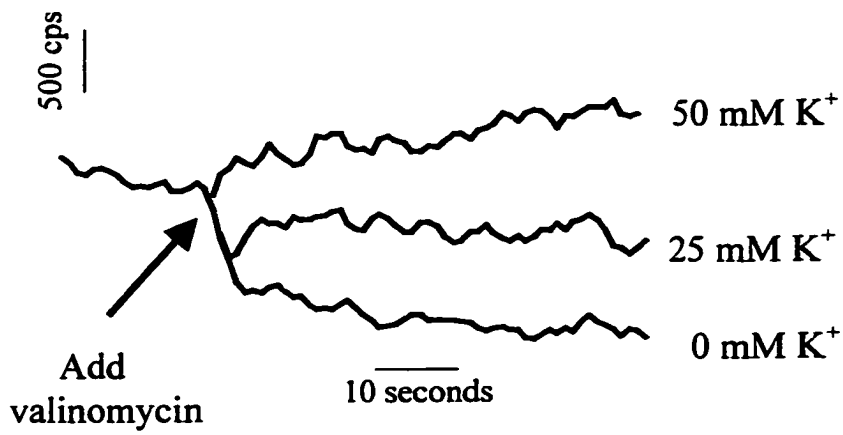

Fig. 4B

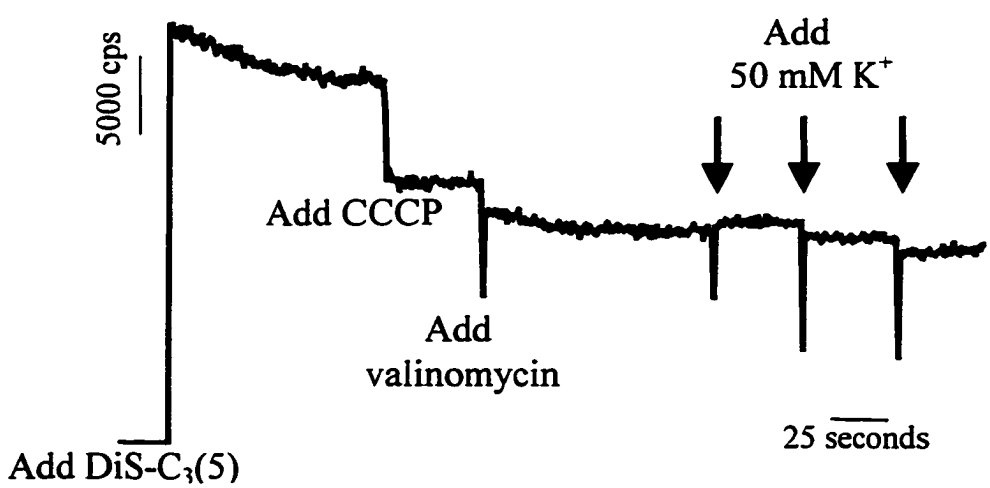

Fig. 5: Effect of Na^+ on membrane potential. 5A: Sperm were suspended in varying concentrations of Na^+ containing CCCP, followed by the addition of DiS-C_3(5) (not shown). Following baseline stabilization, 10 mM monensin was added. A hyperpolarization (downward deflection) is observed in 0-35 mM Na^+, and a depolarization (upward deflection) in 45-50 mM Na^+. 5B: Sperm were suspended in ½ NaF containing CCCP, followed by the addition of DiS-C_3(5) where indicated. Following baseline stabilization, 10 mM monensin was added, followed by the addition of 50 mM increments of Na^+ (arrows). Each downward spike represents the addition of another 50 mM Na. A depolarization is observed upon addition of 50 mM Na^+. The next 50 mM addition resulted in no change in potential. With each additional increment of Na^+ (150-300 mM) a hyperpolarization is observed.

Fig. 5A

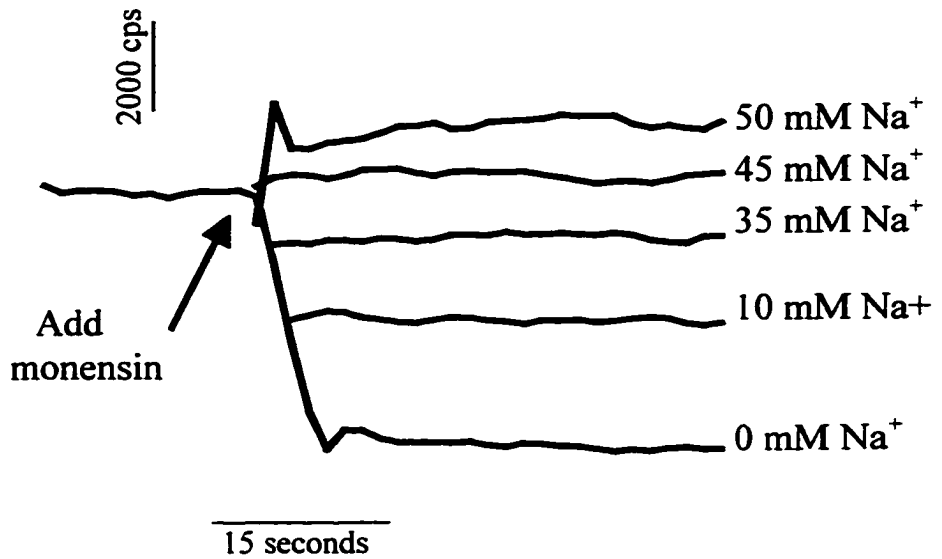

Fig. 5B

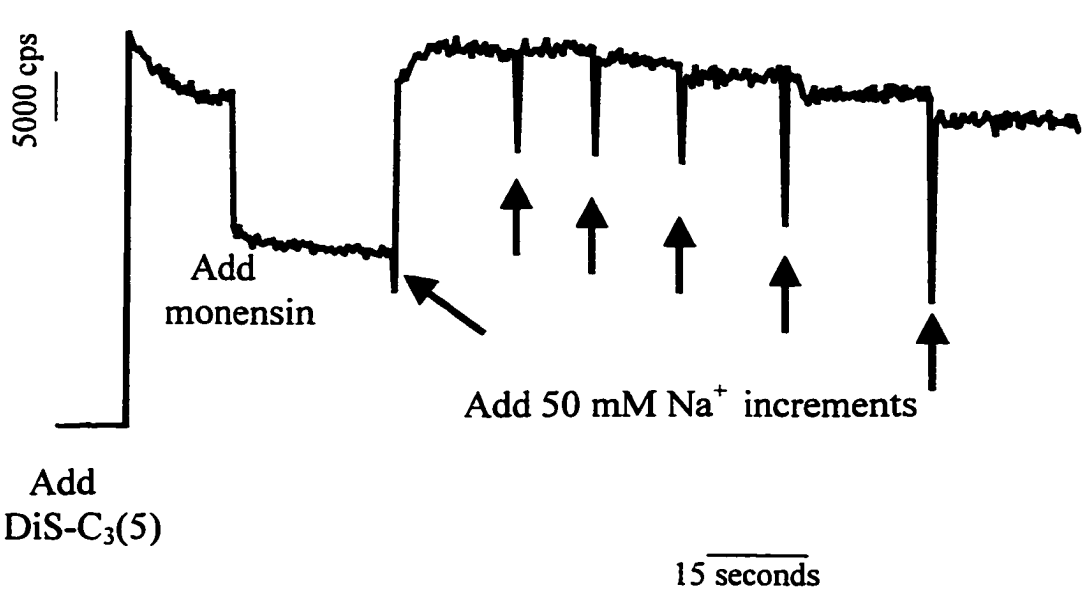

Fig. 6: Effect of SMIF on $[Ca^{2+}]_i$. 6A: Sperm loaded with Fluo-3 (AM) exhibited an increase in $[Ca^{2+}]_i$ (solid line) upon addition of SMIF (arrow), while addition of ½ FSW to sperm suspensions showed no increase in $[Ca^{2+}]_i$ (dotted line). 6B: The SMIF induced increase in $[Ca^{2+}]_i$ is dependent on $[Ca^{2+}]_o$. Addition of SMIF to Fluo-3 (AM) loaded sperm suspended in ½ FSW (containing 5 mM Ca^{2+}) results in Ca^{2+} influx (solid line). Sperm suspended in ½ CaF do not exhibit an increase in $[Ca^{2+}]_i$ until 20 mM Ca^{2+} (5 mM final) is added to the suspension (dotted line).

Fig. 6A

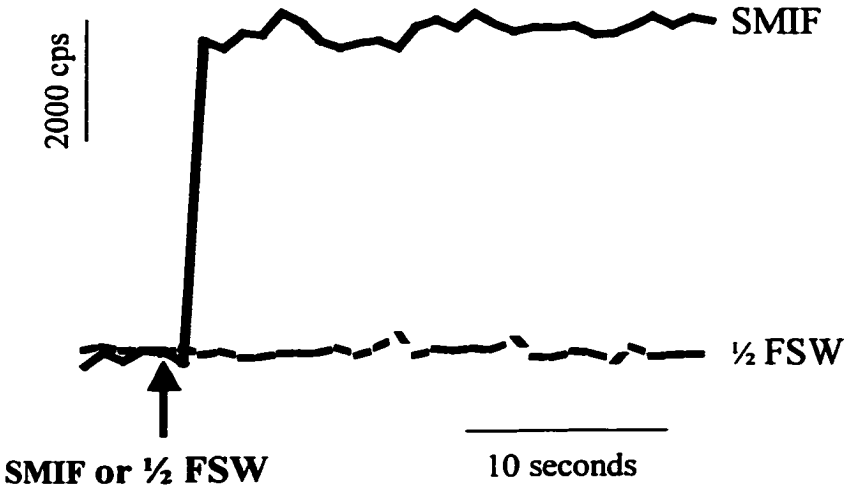

Fig. 6B

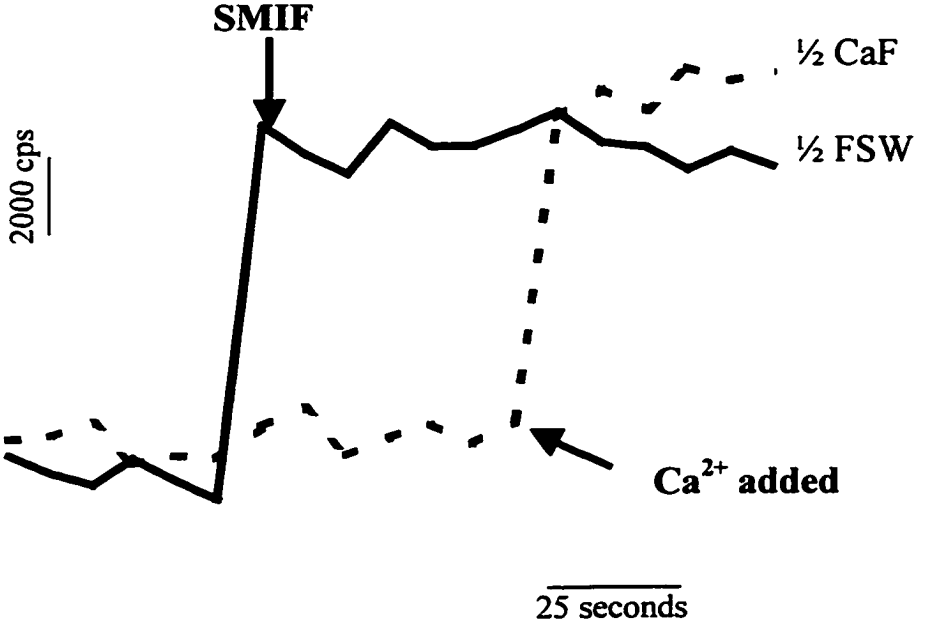

Fig. 7: Effect of Ca^2 inhibitors on the SMIF-induced increase in $[Ca^2]_{i.}$. Sperm loaded with the fluorescent $[Ca^{2+}]_i$ dye, Fluo-3, were suspended in ½ FSW with or without inhibitor, followed by the addition of SMIF (arrow). 10 μM flunarazine (T channel inhibitor) and 100 μM nifedipine (L channel inhibitor) completely inhibited the SMIF-induced influx in Ca^{2+}.

Fig. 7

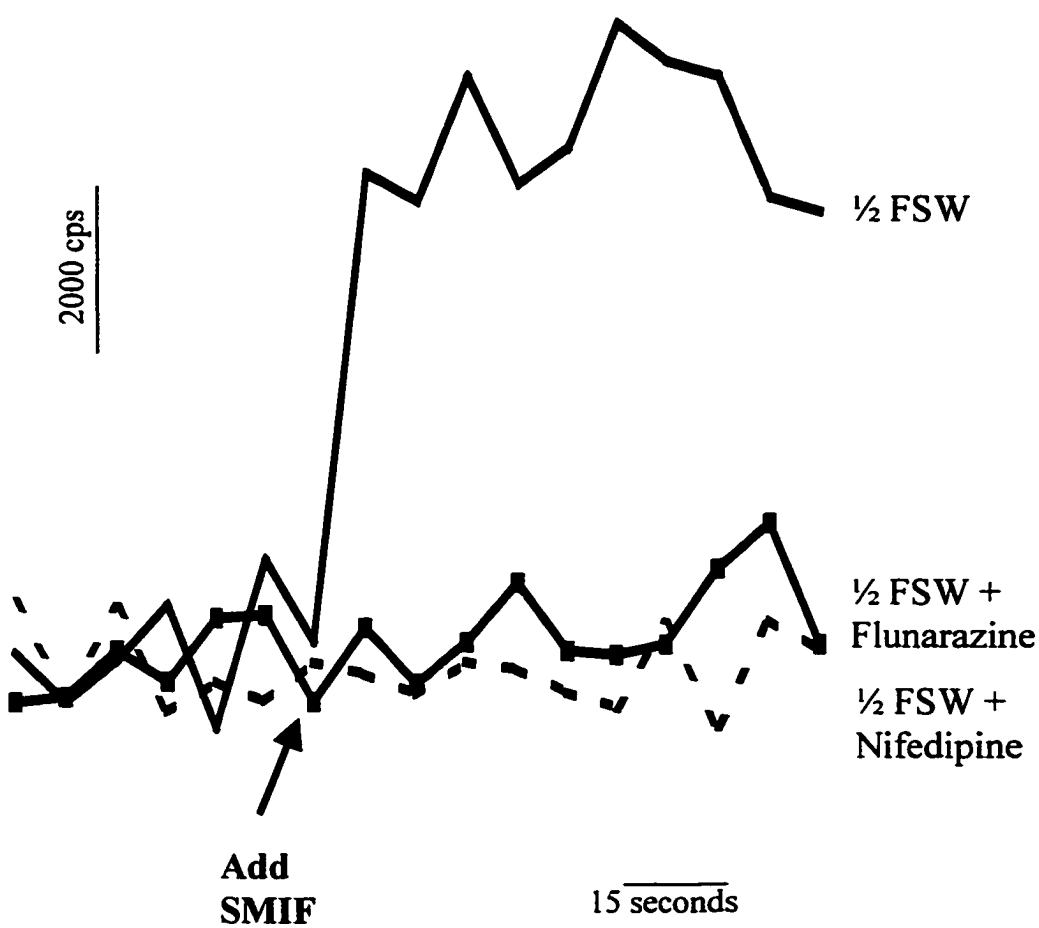

Fig.8: $[Ca^{2+}]_i$ and motility pattern in sperm incubated in 1/2 FSW + SMIF, 1/2 NaF, or 1/2 FSW. The addition of SMIF to sperm incubated in 1/2 FSW (arrow) resulted in a sudden, dramatic increase in $[Ca^{2+}]_i$, while a gradual increase in $[Ca^{2+}]_i$ was observed in sperm incubated in 1/2 NaF. The maximum level of $[Ca^{2+}]_i$ observed in ½ NaF never reached the level of the SMIF-induced increase, reflecting the difference in motility patterns between the two treatments (linear in ½ NaF vs. circular with SMIF). No change in $[Ca^{2+}]_i$ was observed in sperm incubated in 1/2 FSW alone, and sperm remained immotile in this medium.

Fig. 8

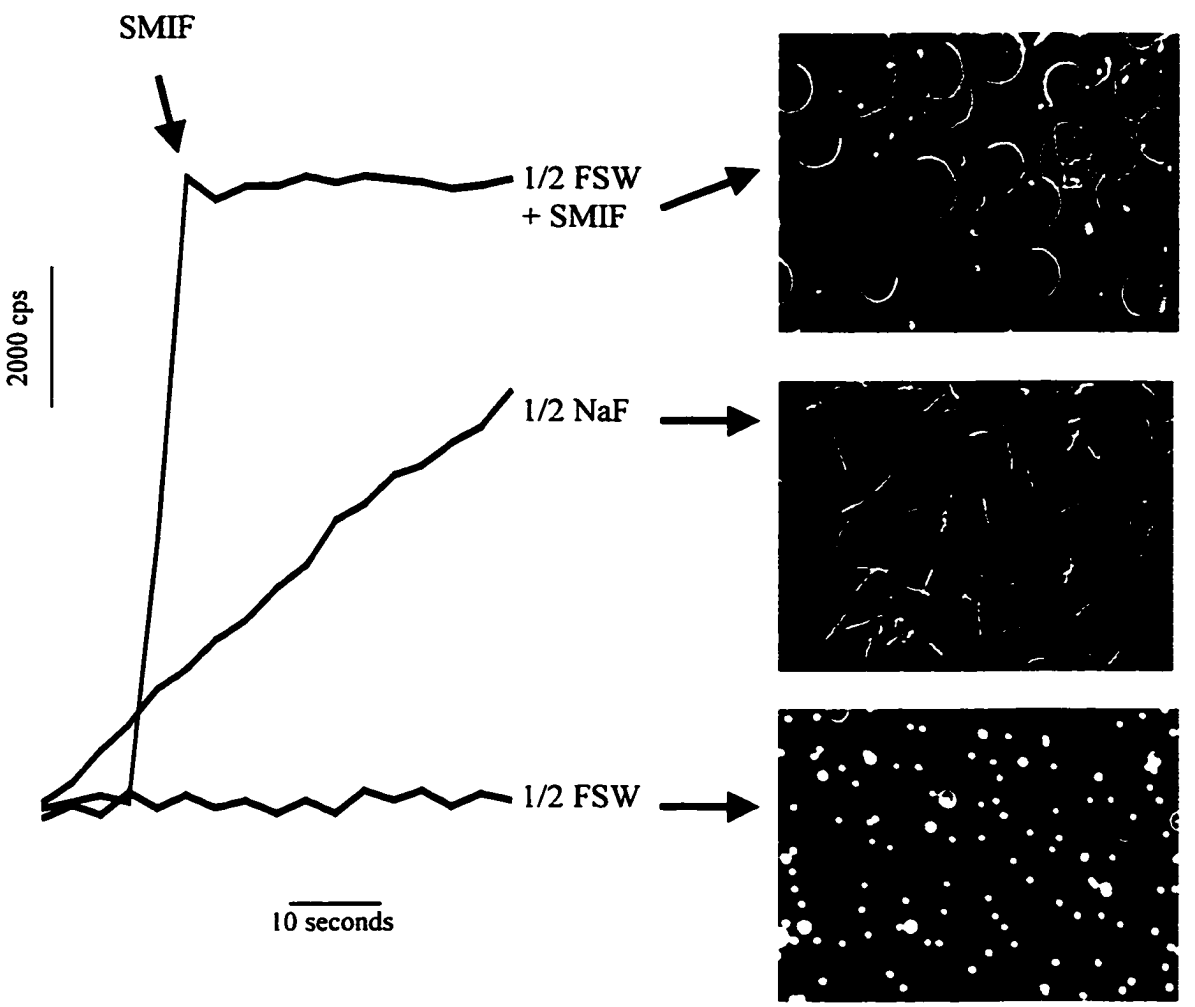

Fig. 9: Efflux of Na^+ in response to the addition of Ca^{2+}. Sperm were suspended in ½ NaCaF containing the extracellular Na^+ probe, NaG. Upon addition of 20 mM Ca^{2+} (5 mM final) at arrow, an efflux of Na^+ was observed (top tracing). This efflux was completely inhibited by 50 uM bepridil (bottom tracing) and partially by 50 uM flunarazine (middle tracing).

Fig. 9

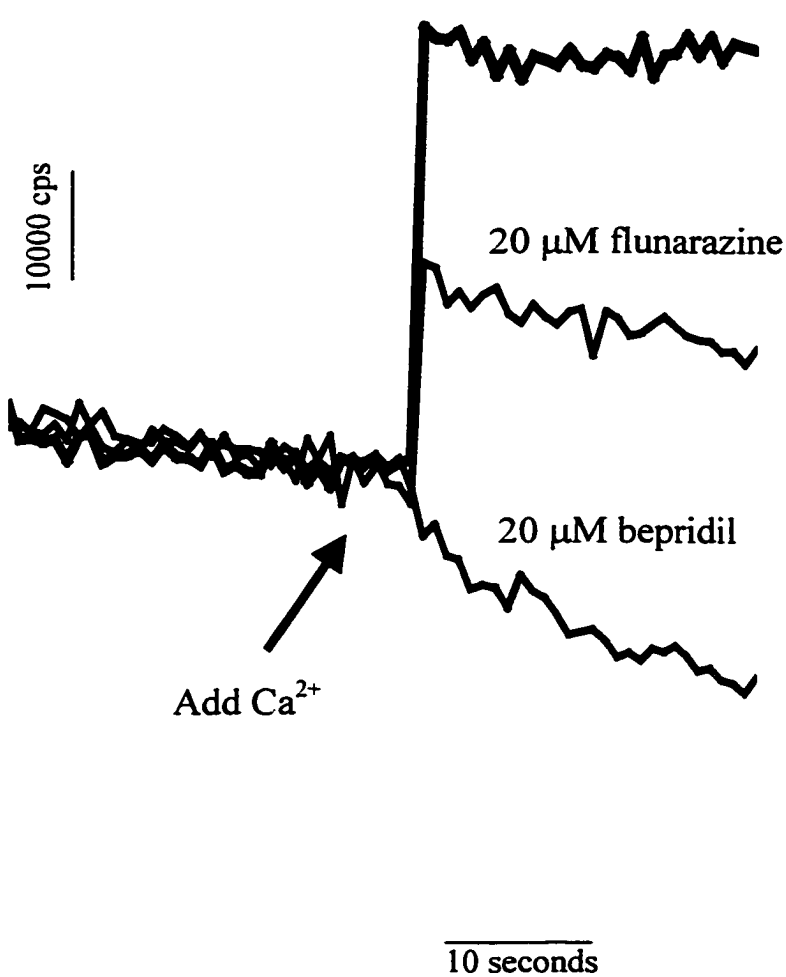

Fig. 10: Effect of SMIF on $[Na^+]_i$. 10A: Sperm loaded with the cell permeant $[Na^+]_i$ fluorescent probe, NaGp were added to suspensions of ½ FSW (top tracing) or ½ FSW containing SMIF (bottom tracing). Sperm suspended in ½ FSW with SMIF exhibited a decrease in fluorescence intensity as compared to sperm suspended in ½ FSW alone. 10B: An average decrease in fluorescence intensity of 12% was observed upon addition of SMIF to sperm suspended in ½ FSW. This was significantly greater ($p=0.008$) than that observed upon addition of ½ FSW alone (1.1% change).

Fig. 10A

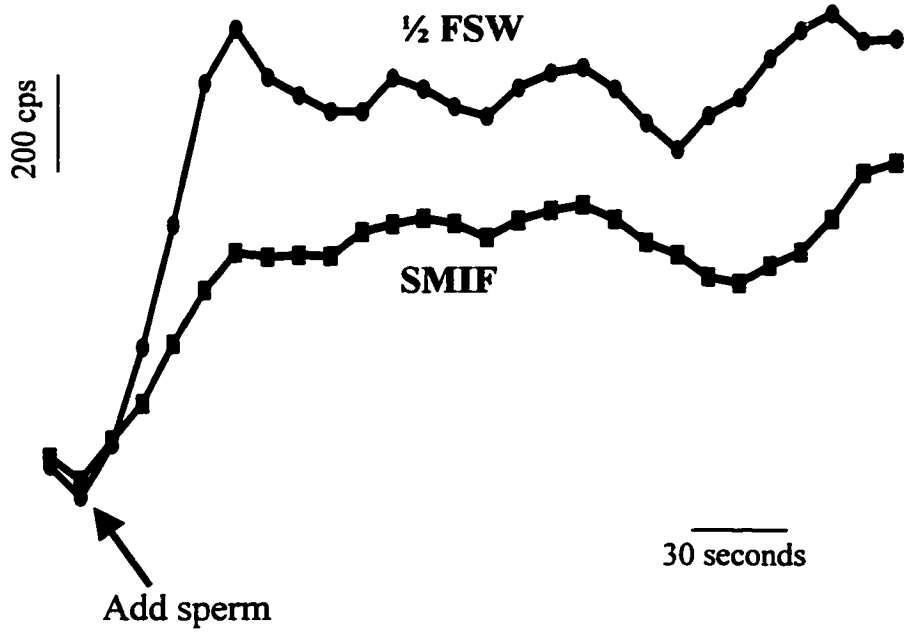

Fig. 10B

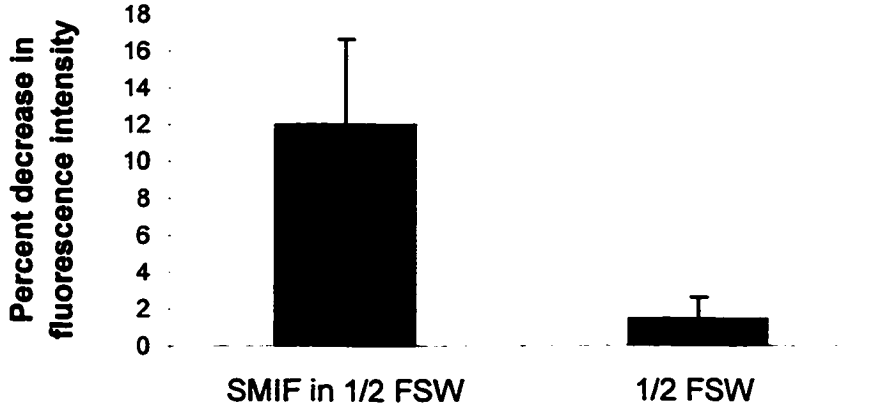

Fig. 11: Effect of PDBu on $[Ca^{2+}]_i$. 10 uM PDBU (arrow) induced an increase in $[Ca^{2+}]_i$ in sperm suspended in ½ FSW (top tracing). This increase was not observed in sperm incubated in ½ CaF (bottom tracing), indicating a dependence on $[Ca^{2+}]_o$.

Table 2: Effect of PDBu on $[Ca^{2+}]_i$ increase. PDBu, in the presence of calphostin, flunarazine, or bepridil, was added to sperm suspended in ½ ASW or ½ CaF and the increase in $[Ca^{2+}]_i$ measured using Fluo-3. PDBu induced an increase in $[Ca^{2+}]_i$ but only when Ca^{2+} was present in the medium. The increase in $[Ca^{2+}]_i$ was inhibited by calphostin, flunarazine, and bepridil.

Fig. 11

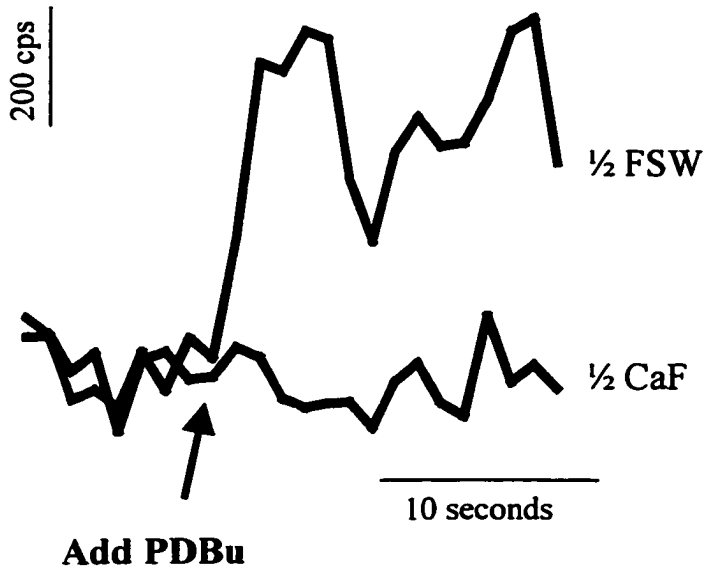

Table 2

Treatment	% Increase in $[Ca^{2+}]_i$	% Inhibition of $[Ca^{2+}]_i$ increase
10 μm PDBu	55.1	NA
10 μm PDBu + 10 μm flunarazine	27.0	51.0
10 μm PDBu + 30 μm bepridil	21.9	60.3
10 μm PDBu + 50 μm calphostin C	22.6	59.0
10 μm PDBu in ½ CaF	0	100

CHAPTER 3

Effects of Creosote-Treated Wood on Development in the Pacific Herring

Introduction

The Pacific herring (*Clupea pallasi*) is a marine teleost that resides in near shore waters over the continental shelf for most of the year, but spawns in bays and estuaries during the winter-spring months (Alderdice and Velsen, 1971: Barnhart, 1988). Males typically spawn first, which then stimulates spawning by females and other males (Stacey and Hourston, 1982). Eggs are spawned onto a variety of substrates, including marine vegetation, rocks, pier pilings and gravel, and development through hatching takes place upon these substrates (Haegele *et al.*, 1981). Freshwater input has been implicated as an important factor in reproduction, at least in some populations of herring, and may actually stimulate spawning in the field (Rabin and Barnhart, 1986). It has previously been shown that elevated ($\geq$28 ppt) and reduced ($\leq$8 ppt) salinities have a profound effect on sperm function (inhibition of motility), fertilization (decrease) and development (reduced hatching rates, abnormal morphology) in the herring, (Griffin *et al.*, 1998). Factors other than salinity also influence the reproductive success of herring as well. Increased water temperatures, such as those encountered during El Nino events, result in a decline in spawning biomass (Spratt, 1992). Other environmental conditions on the spawning grounds, such as storms, water quality, and predation, also contribute to overall reproductive success (Haegele and Schweigert, 1985). Finally, spawning in highly urbanized and industrialized estuaries may expose spawning herring and their progeny to a variety of anthropogenic stressors.

San Francisco Bay is the largest estuary on the west coast of the United States and is surrounded by a large metropolitan area in which urban, commercial, recreational,

agricultural, and industrial activities impact the bay (San Francisco Estuary Project, 1991). It is home to many species of plants and animals, as well as serving as a wintering and staging area for many shorebirds. It also provides important spawning and nursery grounds for a number of fish species, including the Pacific herring (State of California, Department of Fish and Game, 1995). The Pacific herring are not only important ecologically, they also represent the third largest fishery in San Francisco Bay.

Human beings have dramatically altered the character of the bay, especially since the 1850's, as reflected by its primarily urban and industrial usage (San Francisco Estuary Project, 1991). Agriculture has had a profound influence on the bay, including the draining of wetlands, diversion of water for irrigation, and pollution of waters with pesticides and nutrients. In addition to agricultural and urban pollution, industrial and military uses have made San Francisco Bay one of the most polluted estuaries in North America (Nichols, 1995). It is estimated that the surrounding areas discharge up to 40,000 metric tons of pollutants into the bay each year. These pollutants derive from a variety of sources, including agricultural and urban runoff, municipal wastewater discharge, industrial wastewater discharge, and dredging (Nichols, 1995). A diverse array of metals and organics common to urbanized estuaries have been identified in San Francisco Bay, resulting in health advisories for fish consumption (San Francisco Regional Water Quality Control Board, 1995). In addition, man-made structures, such as piers, may modify habitats by the release of pollutants or by altering habitat accessibility or preference.

The gametes and embryos of aquatic organisms are highly susceptible to the effects of natural and anthropogenic stressors. Embryo mortality or malformation is often observed at lower concentrations than those that would affect adult organisms (Bantle, 1985, von Westernhagen, 1988, Manson and Wise, 1991). Early developmental stages may be differentially susceptible to pollutants for a variety of reasons: rapid cell division and differentiation or lack of repair mechanisms may not allow embryos to repair damage, disruption of cell signaling processes required for differentiation of cells or tissues or with migration of cells to form the precursors of adult tissues, lack of detoxification mechanisms, or metabolic costs of DNA repair or detoxification. Lack of mobility also renders embryos more susceptible to pollutants. Herring embryos remain attached to the spawning substrate for 8-10 days prior to hatching, and thus are unable to escape exposure to potentially harmful compounds. Much of the San Francisco waterfront has creosote-treated pilings, and as natural substrates (vegetation) have declined, there has been an increased use of these man-made structures as spawning substrates (Diana Watters, California Department of Fish and Game; personal communication). Observations of embryos spawned onto creosote-treated pilings at one of our field salinity sites (see Chapter 1) indicated that development on these pilings was abnormal. This, together with the increased use of pilings as spawning substrates, provided the stimulus for investigating the effects of creosote on early development in the herring.

The study of the effects of pollutants on organisms in an estuary such as San Francisco Bay is complicated by the complex mixtures of pollutants present in such an

ecosystem. While such mixtures provide a more realistic estimate of environmental exposure and effect, it is more difficult to define the precise role of individual components of the mixture. Laboratory exposures with individual toxicants are useful for determining the mechanism of action of that toxicant. However, the behavior of a toxicant in a mixture may be altered by a number of synergistic or antagonistic processes that fail to correlate with its mechanism of action as a single entity. Coal tar creosote is commonly used as a wood preservative in a variety of terrestrial and aquatic applications, including railroad ties, utility poles, and pilings (von Burg and Stout, 1992). Manufactured through distillation of coal or coal tar, creosote is complex mixture of over 300 known compounds, including polycyclic aromatic hydrocarbons (PAHs), cresols and phenols: the PAH constituents are considered to present the majority of toxicity to organisms (U.S. Public Health Services, 1990; von Burg and Stout, 1992). Creosote, or its PAH constituents have been shown to be cytotoxic, genotoxic, mutagenic, and carcinogenic in a number of organisms, both in the laboratory and in the field (Malins *et al.*, 1985; von Burg and Stout, 1992; Fournie and Vogelbein, 1994a,b; Gagne *et al.*, 1995 Shugart, 1995). However, only limited studies of the effects of creosote-derived compounds on early reproductive events have been undertaken (Peterson *et al.*, 1978; Iyer et al. 1992) particularly on organisms in the marine environment.

In the present study we demonstrate that creosote-derived compounds have a dramatic effect on normal development in the herring, affecting embryonic behavior, cardiovascular function, hatching success, and larval morphology. In addition, the toxic

effects of creosote-derived compounds are enhanced at salinities that are less than optimal for early development.

Materials and Methods

Collection of animals and gametes:

Pacific herring were collected by otter trawl from San Francisco Bay by the California Department of Fish and Game during their annual spawning survey. Fish were kept at 4°C and transported to the Bodega Marine Laboratory within 4 hours of collection. Testes and ovaries were dissected upon arrival at the laboratory as previously described (Griffin *et al.*, 1998), and stored in individual petri plates at 4°C under moist conditions until use. Some herring were collected from British Columbia and Prince William Sound, Alaska by researchers at the Department of Fisheries and Oceans, Biological Sciences Branch, Nanaimo, British Columbia and the Alaska Department of Fish and Game, Cordova, Alaska. The gonads were dissected and shipped overnight at 4°C under moist conditions to the Bodega Marine Laboratory.

Assessment of hatching and normal development

Hatching success was determined by counting the number of empty chorions (egg shell membranes) versus the number of chorions containing unhatched embryos, and was evaluated when controls showed >80% hatching. Normal larval morphology was evaluated as previously described (Griffin et al. 1998), and was characterized by the presence of straight spines, and absence of pericardial or yolk sac edema.

Materials:

Seawater was filtered through 0.45 μm Nalgene filters (Fisher Sci. Pittsburgh, PA) and diluted with distilled water (dH_2O) to prepare ½ filtered seawater (1/2 FSW) which is the optimal salinity for fertilization (Griffin *et al.*, 1998), and is ~16 parts per thousand (ppt) salinity. 8 parts ppt and 28 ppt seawaters were prepared by diluting with the appropriate volume of dH_2O. The pH and salinities of all solutions were verified at the beginning and end of each exposure. Creosote-treated wood, at or below water level, was obtained from creosoted pilings located at the Fort Baker Yacht Club in Sausalito, CA. These pilings were estimated to be approximately 40 years of age. Untreated-wood (kiln-dried Douglas fir) was used as a reference material in experiments. Creosote-treated wood and untreated wood were conditioned statically in ½ FSW for 5 days prior to use. Commercial creosote was a generous gift from J.H. Baxter Wood Preserving (Eugene OR). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation and analysis of seawaters containing creosote-derived compounds

Water samples from acute embryo exposures in ½ FSW, ½ FSW containing untreated-wood, and ½ FSW containing creosote-treated wood were collected daily, filtered to remove particulate matter, and divided into 2 aliquots for spectrofluorometric analysis and GC-MS. For consistency, the ratio of wood to ½ FSW was 1 g/200 ml. Samples were stored in acid-washed amber bottles and frozen at -20°C until analysis.

The excitation spectra for all water samples were collected using a spectrofluorimeter (Photon Technologies, Inc.), scanning between 220-340 nm (emission 353 nm) in 1 cm quartz cuvettes (modified from Villaizan *et al.*, 1995). Peak excitation intensities were compared to the commercial coal-tar creosote formulation, and based on

a standard curve using this formulation, the samples were assigned nominal concentrations.

For determination of the LC_{50} and investigation of the sublethal effects of creosote-derived compounds, creosote-treated wood was incubated for 24 hours at 4°C in ½ FSW (1g/200 ml), followed by filtration to remove particulate matter. Serial dilutions of the resulting water samples containing creosote-derived compounds (nominal concentrations of 0.0015-1.5 mg/L based on fluorometric analysis) were prepared in ½ FSW.

For GC-MS analysis, samples were filtered through C-18 bond elute columns (Analytical International), previously conditioned by rinsing with methanol, hexane, dH_2O, and ½ FSW (Facility for Advanced Instrumentation). 50 ml of each water sample were loaded onto each column, and withdrawn (5 ml/min) using a Hamilton syringe. The filtrate was retained for bioassay. The C-18 filters were washed with dH_2O, and eluted with hexane at a flow rate of 1 ml/min using a Hamilton syringe. Samples were transported to the Facility for Advanced Instrumentation (U.C. Davis) for analysis.

Assessment of the effects of creosote-treated pilings on naturally spawned embryos

Embryos were collected from the site of a natural spawning event (Presidio Yacht Club, Fort Baker, CA) approximately 2 days prior to hatch. Two locations at the site were sampled: the surface of a creosote-treated piling, and PVC pipe located approximately 1 foot away from the piling. Samples were collected and transported in ambient seawater to the Bodega Marine Laboratory, where they were incubated in

ambient seawater at 13°C until hatching. Hatching success and morphology were assessed as described above.

Laboratory exposure to creosote-treated wood

Herring eggs (150-200) were deposited into acid-washed glass finger bowls (200 ml), fertilized in ½ FSW, and rinsed in ½ FSW to remove sperm (Griffin et al. 1998). Embryos were exposed to ½ FSW (200 ml), untreated-wood (1.0 g/200 ml ½ FSW) or creosote-treated wood (1.0 g/200 ml ½ FSW), and incubated at 13°C in a temperature-controlled incubator with daily water changes until hatching. Embryos were examined daily using an Olympus stereo-zoom microscope and monitored for progression of normal development. Unfertilized eggs or embryos that failed to develop were removed to prevent contamination. Heart rates and embryonic activity were recorded using an Olympus CCD camera and video recorder (Panasonic). Hatching success and morphological deformities were assessed as described above.

Exposure to commercial coal-tar creosote and C-18 extracted water and determination of the LC_{50}

Approximately 100 eggs were fertilized on acid-washed glass slides, which were placed into shell vials (20 ml). Stock solutions (0.1 and 1.0 g/L) of the commercial coal-tar creosote formulation were prepared in dimethyl sulfoxide (DMSO). Embryos were exposed to one of the following treatments: ½ FSW, solvent control (0.005% DMSO in ½ FSW), 0.1 or 1.0 mg/L commercial coal-tar creosote, C-18 extracted ½ FSW, or ½ FSW containing creosote-derived compounds prior to C-18 filtration (1.5 mg/L). Hatching and larval morphology were assessed as described above.

Hatching success was used to determine the LC_{50} of ½ FSW containing creosote-derived compounds. Eggs were fertilized on acid-washed glass slides in shell vials (20 ml) and incubated in varying concentrations of creosote-derived compounds in ½ FSW (0.0015-1.5 mg/L nominal) or in ½ FSW (control). Hatching success was assessed as described above.

Combined effects of creosote-derived compounds and salinity:

Based on the results of the experiment determining the LC_{50}, a sublethal concentration of creosote-derived compounds (0.003 mg/L), resulting in hatching rates within 5-10% of the ½ FSW control, was selected to investigate the combined effects of salinity and creosote-derived compounds. Fertilized embryos on glass slides were incubated in 8, 16, or 28 ppt seawaters with or without creosote-derived compounds (0.003 mg/L) at 13°C with daily water changes. Embryonic heart rate, hatching success, and larval morphology were evaluated as described above.

Statistical Analysis:

Samples collected from the field were not analyzed for statistical significance due to low sample size, and are reported only in comparison to the control. For laboratory exposures, data were expressed as means $\pm$ SD of three experiments with three replicates per treatment group within each experiment. Differences between treatment groups were analyzed by one or two way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons (SigmaStat; Jandel Scientific, San Rafael, CA). The LC_{50} was determined using ToxCalc (Tidepool Scientific Software, Mckinleyville, CA).

Results:

Effects of creosote-treated pilings on herring development in the field

When creosote-treated pilings were used as spawning substrate, a dramatic effect on herring embryos was seen. None of the embryos maintained on the creosote-treated wood survived to hatching. However, embryos removed from the creosote-treated piling two days prior to hatch exhibited a 24% hatch rate when compared to the hatching success (86%) of cohorts collected from the surface of a PVC pipe located approximately 1 foot away from the piling (Fig. 1) indicating the inhibitory effect of creosote-treated wood. All of the embryos removed from the piling died shortly after hatching, and each exhibited varying degrees of morphological abnormalities, particularly scoliosis of the spine. In contrast, >95% of larvae from the PVC pipe were free of these abberations. These observations were the stimulus for examining the lethal and sublethal effects of creosote-derived compounds in the laboratory, as well as a possible interaction with another stressor, salinity.

Laboratory Exposures

Utilizing a standard curve generated using the commercial coal-tar creosote, the nominal concentration of creosote-derived compounds in the water samples (n=15) was determined to be 1.2 +/- 0.3 mg/L. The fluorescence spectra of the water samples were consistent with that of the commercial coal-tar formulation, with a peak intensity at approximately 290 nm (Fig.2).

Exposure of herring embryos to 200 ml of 1/2 FSW containing 1 g of creosote-treated wood, resulted in arrested development in a large proportion of embryos (40-50%) within the first 2-3 days of exposure. These effects were most pronounced for embryos adhering directly to the surface of the wood, with all of these embryos failing to develop. As such, all data presented below are for embryos not adhering to the creosote-treated wood. A dose response curve (Fig 3) was generated using nominal concentrations calculated from a standard creosote curve and the LC_{50} determined to be 0.182 mg/l.

Development:

1/2 FSW control embryos exhibited a normal pattern of development with optic vesicle pigmentation occurring at days 4-5 of incubation (Fig. 4a). The embryos adhering to untreated-wood, or exposed to its water-borne compounds, underwent normal development (Fig 4b), although they were more difficult to observe due to their darker appearance (possibly due to staining of the chorions by the wood-derived compounds). Development of embryos exposed to creosote-derived compounds (1.2 mg/L) was typically delayed, as evidenced by a lack of optic vesicle pigmentation (Fig 4c) until 6-7 days after fertilization. In addition, exposed embryos exhibited a variety of abnormalities. Mortality during the first 2 days of incubation was one of the most consistent observations, and was frequently associated with the development of prominent yolk vacuoles (Fig 4d).

Heart rate

The heart rates of embryos exposed to creosote-derived compounds were significantly lower than both the 1/2 FSW controls and embryos incubated with untreated

wood ($p<0.05$). Heart rates of embryos incubated in 1/2 FSW and with untreated-wood tended to increase from day 5 to day 9 post fertilization (Fig. 5a), while those of embryos exposed to creosote-derived compounds decreased as development progressed. Heart rates of embryos exposed to untreated-wood were on average 11.5% lower than that of 1/2 FSW controls, however this was not statistically significant ($p>0.05$). By day 9, heart rates of embryos exposed to creosote-derived compounds were 93% lower than the both the 1/2 FSW and embryos exposed to untreated-wood. Over the five-day period, heart rates of embryos exposed to creosote-derived compounds also decreased from 50 beats/min to 9 beats/min. In addition, exposed embryos exhibited moderate to marked arrhythmia (Fig 5b), with cessation of heartbeats ranging from a few seconds to greater than one minute. A pattern of heart rate consisting of slowing heartbeats, cessation of beating, and then a series of rapid heartbeats was observed in several cases. In contrast, cardiac rhythm in the control embryos was consistently rhythmic, with no observed cessation of heartbeats.

Embryonic activity:

Embryos exposed to creosote-derived compounds exhibited both an increased frequency of embryonic activity and an alteration in the type of movement. 1/2 FSW control and embryos exposed to untreated-wood were observed to undergo periodic movements within the chorion starting at about day 4-5, with the frequency decreasing from 6-8 movements/min on day 6 to 1-2 movements/min on day 10 (Fig 6). These movements consisted of vigorous tail or trunk displacements in which embryos twisted 45-360 degrees within the chorion. Initially, embryos exposed to creosote-derived

compounds (1.2 mg/L) exhibited fewer movements (1-2 movements/min on day 5), but by day 9, the frequency of activity was significantly increased ($p<0.05$) to 15-23 movements/min. In contrast to the vigorous movements observed in the controls, exposed embryos exhibited a pattern of twitches or tremors, which in some cases were sustained during the entire observation period. By day 9, only abnormal embryonic activity was observed in embryos exposed to creosote-derived compounds.

Hatching and larval morphology

Larval hatching was affected by creosote-derived compounds and to a lesser extent, by untreated-wood. Hatching rates of control (1/2 FSW) embryos were 88.8% (Fig. 7), while hatching rates for embryos incubated with untreated-wood (73.1%) as well as the creosote-derived compounds (9.0%) were significantly reduced ($p<0.05$). However, in comparison to untreated-wood, exposure to creosote-derived compounds was also significantly reduced (87.6% lower).

Normal larval morphology in 1/2 FSW controls, as evidenced by straight spines, and normal appearing yolk sacs (fig 8 a,d) was typically >90%. All of the hatched larvae, exposed as embryos to creosote-derived compounds, exhibited severe morphological deformities, particularly varying degrees of scoliosis (fig 8b). Embryos exposed to creosote-derived compounds also had a higher incidence of partial hatching, (15-20%) in which initiation of hatching was not followed by complete escape from the chorion (Fig 8c). Pericardial and/or yolk sac edema was observed in ~15% of hatched larvae (Fig 8e). Although larvae from embryos incubated with untreated wood had a

greater incidence of abnormalities, these differences were not significant, and most larvae possessed straight spines, and no evidence of pericardial or yolk-sac edema (fig 8f).

Effect of exposure to varying salinities and creosote-derived compounds

Embryos incubated in both 8 or 28 ppt salinities without creosote had significantly lower hatching rates ($p<0.05$), 34.4% and 26.1% respectively, than in 16 ppt salinity (Fig 9). Normal morphology was also significantly decreased ($p<0.05$) by 34% for both salinities. Addition of sublethal concentrations of creosote-derived compounds (0.003 mg/L) to 8, 16, and 28 ppt salinity waters resulted in lower hatching rates as compared to 8, 16, or 28 ppt salinities alone. For 8 ppt, creosote-derived compound exposure resulted in a 32.7% decrease in hatching success, with a 24% decrease in the number of normal larvae. Exposure to creosote-derived compounds in 16 ppt resulted in a 19.1% reduction in hatching, and a reduction in the number of normal larvae of 34.5%. Embryos exposed to 28 ppt salinity with creosote-derived compounds exhibited a 14.1% reduction in hatching, and a 10.2% decrease in the number of normal larvae. An additive effect of creosote-derived compounds on hatching was observed for both the 8 and 28 ppt treatment groups ($p<0.05$), and for the 8 and 16 ppt treatment groups for morphology..

When the concentrations of creosote-derived compounds were reduced, a reduction in the severity of cardiovascular effects was seen (Fig. 10). Salinity alone significantly decreased heart rates ($p<0.05$) by 21.8% (8 ppt) and 24.6% (28 ppt) as compared to the 1/2 FSW controls. Exposure to creosote-derived compounds resulted in further decreases in heart rate of 27.6%, 24.5% and 24.8% for 8, 16, and 28 ppt, respectively.

C-18 chromatography of creosote-derived compounds and exposure to commercial coal-tar creosote

Extraction of creosote-derived compounds from solution (1g piling/200 ml 1/2 FSW) through a C-18 bond elute column resulted in removal of the majority of toxicity. Embryos exposed to the filtered water had comparable hatch rates to the 1/2 FSW controls, 89% versus 91% respectively, with 87% of the embryos appearing normal as compared to 91% in the controls (Fig 11). As expected, most of the embryos (93%) exposed to non-extracted ½ FSW containing creosote-derived compounds (1.5 mg/L) died during the first few days of incubation.

Embryos exposed to 0.1 and 1.0 mg/L of the commercial coal-tar formulation had significantly lower hatch rates as compared to both the 1/2 FSW controls and C-18 filtrate exposed embryos, with a reduction in hatching of 70.0% and 79.9% respectively. Normal morphology was reduced by 56.0% and 88.6% in these concentrations as well.

Analysis of water samples

Qualitative analysis of the GC-MS spectrum (Facility for Advanced Instrumentation) indicated the presence of large quantities of PAHs (anthracene, and possibly phenanthrene and diphenylethyne), as well as smaller quantities of O-heterocyclics (furans) and straight or branched chain substituted hydrocarbons (hexane, hexanone, hydroxy nonanone, pentanol, butane, octeneone). Substantial amounts of phthalate esters were also present in the sample.

Discussion

Exposure to creosote-derived compounds has significant effects on early development in the Pacific herring. Arrested development during the first few days of incubation was the earliest abnormality detected, manifested as degeneration of embryos and formation of vacuoles in the yolk sac. Those embryos that continued to develop exhibited numerous abnormalities, including delayed optic vesicle pigmentation, cardiac arrhythmia, decreased heart rate, and abnormal embryonic activity, consisting of recurrent and sustained tremors. Hatching success in exposed embryos was significantly decreased, and all hatchlings exhibited severe morphological deformities, including scoliosis, pericardial and/or yolk sac edema. All larvae from creosote-derived compounds-treated wood exposures died shortly after hatching. Similar effects have been observed in herring embryos exposed to other petroleum products. Middaugh *et al.*, (1998) found that exposure to biodegraded crude oil resulted in reduced heart rate, decreased hatching, and gross morphological abnormalities, including scoliosis, pericardial edema, and cranio-facial abnormalities in herring embryos. Kocan *et al.*, (1996) found that herring embryos collected from Prince William Sound three years after the Exxon Valdez oil spill also had lower hatching rates and a higher incidence of morphological deformities. Embryos exposed to untreated-wood also had significantly lower hatching rates as compared to 1/2 FSW controls, however these rates were still significantly higher than those of embryos exposed to creosote-derived compounds. Future investigations should include filtering water from the untreated-wood samples

through C-18 columns, and analysis of the filtrate and retentate to determine the basis of the toxicity exhibited by untreated-wood.

The effects of exposure to creosote-treated compounds were dependent on concentration and proximity to the source. Embryos adhering to creosote-treated wood manifested the most severe effects, with all of the embryos failing to develop beyond the first few days, while many embryos located within a few inches of the wood continued to develop. This effect was observed in both the field and laboratory exposed embryos. Despite continued development, however, hatching rates and normal larval morphology were significantly lower than in unexposed embryos.

Exposure to a sublethal concentration of creosote-derived compounds (3 μg/L) resulted in substantial improvement in heart rate, hatching success and normal larval morphology. Dilutional effects may not be relevant in the environment, at least for embryos adhering directly to the surface of creosote-treated pilings that are likely to experience sustained, high levels of creosote-derived compounds. However, depending on tidal conditions and water flow, dilutional effects could provide some protection for embryos located farther away from pilings. This may account for the difference in hatching rates observed in the laboratory versus the field. The hatching rate of embryos exposed following a natural spawning event was 24% versus 9% for the laboratory-exposed embryos. It is possible that tidal fluxes at the spawning site attenuated the effects of exposure to the pilings, whereas exposures in the lab were conducted statically.

C-18 columns are commonly used for the separation of relatively non-polar organic compounds (e.g. hydrocarbons) (Willard *et al.*, 1988). In this study, the

extraction of seawater containing creosote-derived compounds through the column resulted in total loss of toxicity. Qualitative analysis and identification of the compounds retained by the column by GC-MS indicated the major constituents present are PAHs (anthracene) as well as furans, known constituents of creosote (US Public Health Service, 1990; Hale and Aneiro, 1997), as well as smaller amounts of straight chain hydrocarbons. Not surprisingly, phthalate esters, common contaminants of water and soil (Menzer, 1991) were also detected. Future investigations would need to address the potential effects of these compounds on developing organisms.

It is estimated that the creosote-treated pilings from which our wood samples were obtained were placed into the cove at Fort Baker Marina in the 1950's, and have therefore undergone substantial weathering. Creosote is not appreciably water soluble, and at least some of the lower molecular weight, water-soluble and volatile constituents tend to dissipate. This has led to estimates that the aquatic half-life of creosote is less than 1 week (Borthwick and Patrick 1982). The less water-soluble and higher molecular weight constituents, such as many of the PAHs, tend to accumulate in sediments and particulate matter, presenting the possibility of future contamination as these sediments are disturbed (McElroy *et al.*, 1989, Sved *et al.*, 1997). Since the octanol-water partition coefficients (K_{ow}) for PAHs are relatively high (10^3-10^7), the potential for accumulation of these compounds in organisms is substantial (Rand, 1995), and in fact, PAHs have been detected in barnacles and mussels growing on creosote pilings (Dunn and Stich, 1975,1976) It is somewhat surprising that anthracene was still present in large quantities in our water samples, since this PAH is reportedly readily biodegraded and susceptible to

UV photolysis (Hale and Aneiro, 1997). One possible explanation is that removal of pieces of wood from the creosote-treated pilings led to the release of creosote-derived constituents trapped in pockets of the wood, resulting in enhanced toxicity in our laboratory tests. While this may be true, it has not been tested. The fact remains that embryos collected from undisturbed creosote-treated pilings also manifested similar effects as did exposed embryos in the laboratory.

The combined effects of salinity and exposure to certain toxicants have been previously documented in herring. Von Westernhagen et al. (1974) demonstrated that reduced salinities enhanced the toxicity of cadmium to herring embryos, resulting in decreased hatching rates and increased larval malformations. In agreement with previous studies (Griffin *et al.*, 1998), altered salinities (8 and 28 ppt) resulted in decreased hatching success and an increase in abnormal larval morphology. In addition, exposure to both creosote-derived compounds (3 μ/L) and 8 or 28 ppt salinities resulted in significantly lower hatching rates and a higher incidence of abnormalities than embryos exposed to creosote-derived compounds or altered salinities alone. Alterations in salinity or exposure to creosote-derived compounds alone resulted in significant reductions in heart rate as well; and a general trend towards further reduction in heart rate in embryos exposed to a combination of creosote-derived compounds and altered salinities was observed.

The mechanisms by which creosote-derived compounds exert their toxic effects in herring are not known. The presence of significant numbers of degenerating embryos during the first few days of development suggest that creosote, or its PAH constituents,

may have a cytotoxic effect on the developing embryo. Cytotoxic effects on rainbow trout hepatocytes have been shown to be correlated with certain PAH constituents of creosote, including anthracene (Gagne, *et al.*, 1995). It is also possible that DNA damage early in the developmental program may result in the embryolethality observed in this study, since creosote (particularly its PAH constituents) have been shown to induce point mutations, chromosomal damage, and lethal damage to DNA in rainbow trout hepatocytes (Gagne, *et al.*, 1995) and mouse tissues (Randerath, *et al.*, 1996). Induction of cytochrome P4501A in channel catfish as also been shown to result in binding of reactive metabolites of aminoanthracene to DNA (Watson *et al.*, 1998).

The cardiac abnormalities observed suggest a different mechanism of action, perhaps later in development. Pericardial edema or blue sac disease has been observed in a number of fish embryos exposed to polyhalogenated compounds, including lake trout (Spitsbergen *et al.*, 1991), fathead minnow and killifish (Olivieri and Cooper, 1995), rainbow trout (Walker *et al.*, 1992), medaka (Wisk and Cooper, 1990) and zebrafish (Henry *et al.*, 1997). It has been suggested that cytochrome P4501A (CYP1A) induction in endothelium results in alteration of endothelial tissues leading to edema (Guiney *et al.*, 1996). PAHs are known inducers of CYP1A (Di Giulio, *et al.*, 1991), thus it is possible that the cardiovascular effects observed in herring embryos in response to exposure to creosote-derived compounds may be due to the same mechanism. It is not known if CYP1A expression is present in herring embryos during any developmental stages, thus it would be useful to investigate this possible mechanism for the cardiovascular effects observed.

In the zebrafish, slowed heart rate in dioxin exposed embryos was associated with significant edema of skeletal muscle and head, as well as loss of blood flow to these tissues (Henry *et al.*, 1997). The incidence and frequency of tremors in creosote-derived compounds-exposed embryos may reflect a similar defect, such that neurological control of muscle is affected. This may also explain the appearance of scoliosis in hatched larvae. If vigorous muscular contractions within the chorion are required for efficient exercise of the embryos, lack of this activity may result in atrophy or failure of muscle to develop normally. Additionally, this may also account for the increase in the incidence of partially hatched larvae, which may lack the necessary muscular power to break free of the chorion.

The overall impact of exposure to creosote-derived compounds on Pacific herring populations is not known. Herring embryos deployed in Prince William Sound three years after Exxon Valdez oil spill also had lower hatching rates and a higher incidence of morphological abnormalities than embryos collected from unoiled sites (Kocan *et al.*, 1996), suggesting that long term effects of petroleum products can impact herring populations, at least at the local level. In San Francisco Bay, the loss of preferred spawning habitat, i.e. macroalgae, may result in increased spawning onto other less suitable substrates, such as pilings, and in fact herring have been observed to spawn on creosote-treated pilings along the San Francisco waterfront (Diana Watters, Personal communication). It has been theorized that spawning herring may show some degree of homing behavior (Hourston, 1982). If this is the case, one might expect a decline in

herring spawning biomass if herring continue to return to spawn onto less suitable substrates in subsequent years.

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Fig. 1: Effect of creosote wood on hatching success and normal larval morphology from a natural spawn. Control embryos collected from chambers 1 foot away from the piling had a >90% hatching rate with >90% of the larvae appearing normal. Hatching rates for embryos collected from the creosote-treated piling had significantly lower hatching rates, and all of the larvae exhibited abnormal morphology and died shortly after hatching.

Fig. 1

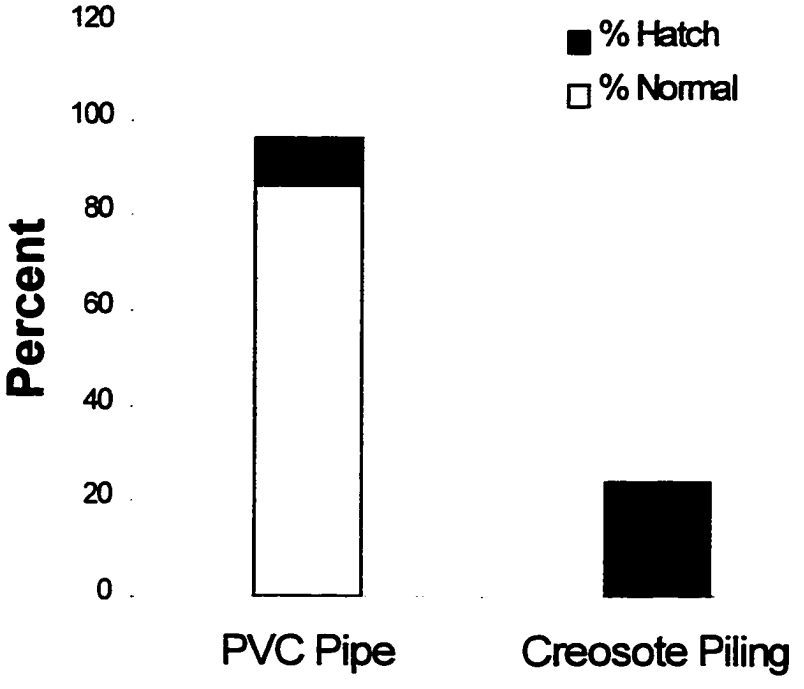


Fig. 2: Excitation scan of commercial coal-tar creosote, incubation waters from embryos exposed to creosote-derived compounds, and incubation waters from embryos exposed to untreated-wood compounds. A peak intensity is seen at approximately 290 nm (characteristic for PAH's). Based on the standard curve, the concentration of the creosote-derived compounds in the water sample was 1.2 mg/L. The characteristic peak at 290 nm was not detected for the untreated-wood sample.

Fig. 2

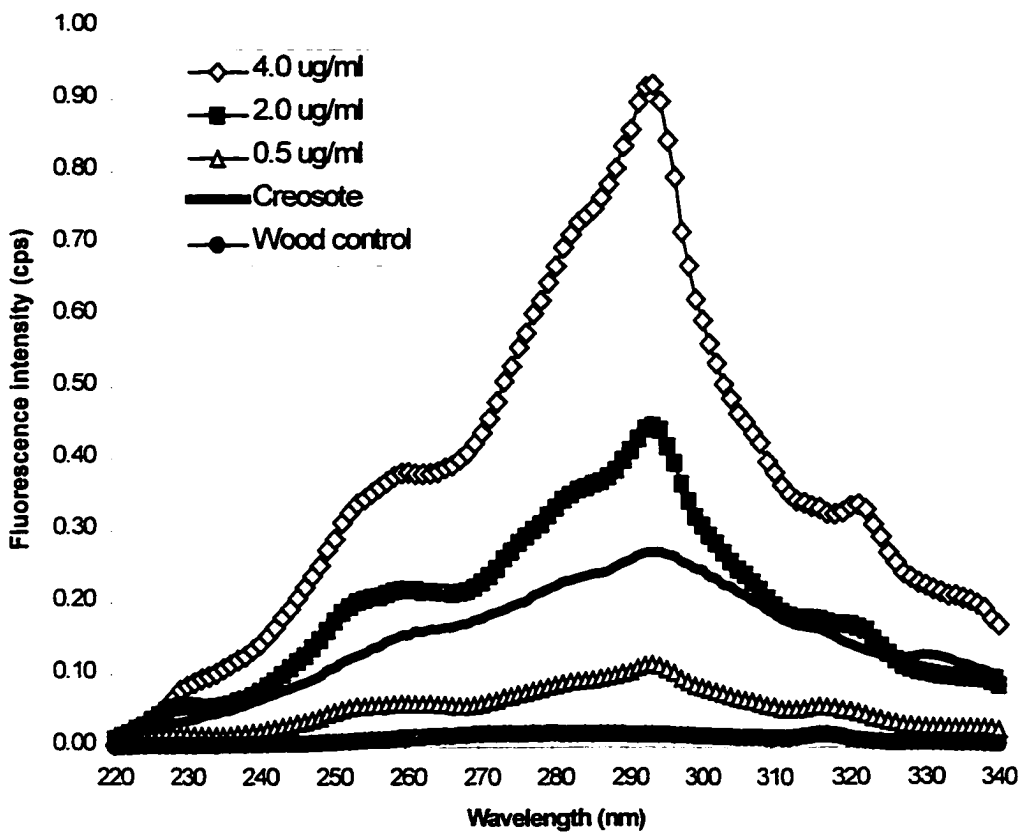


Fig. 3. Dose response curve for creosote-derived compounds. Hatching success was evaluated for embryos incubated in varying concentrations of creosote-derived compounds as compared to controls in ½ FSW. The LC_{50} was determined to be 0.182 mg/L

Fig. 3

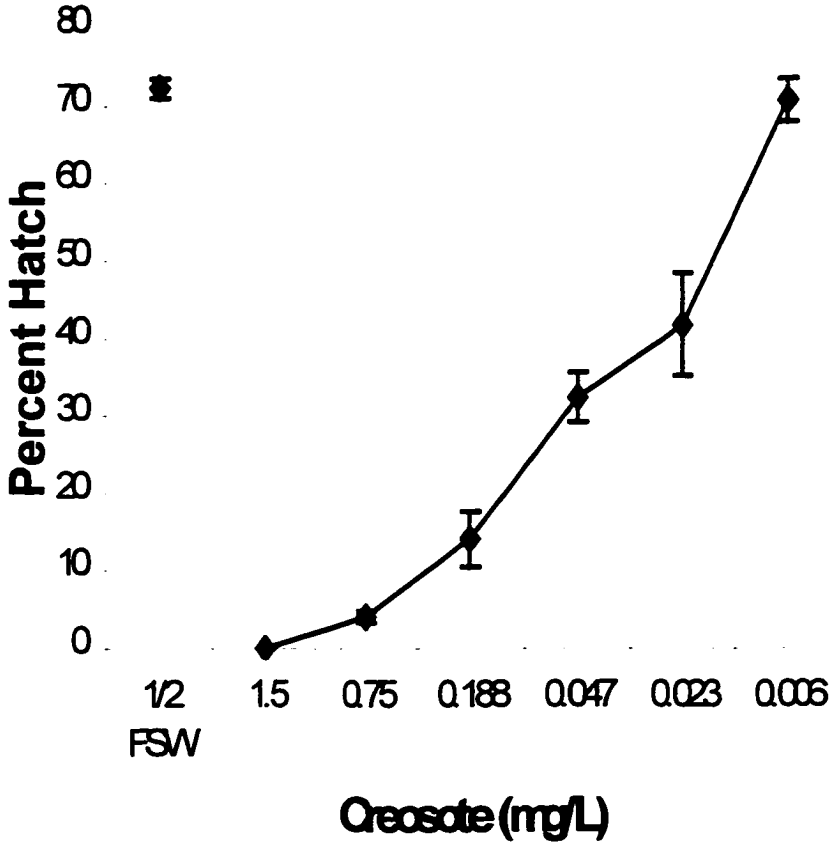


Fig. 4: Effect of creosote-derived compounds on early development. Embryos were exposed to ½ FSW, ½ FSW with untreated-wood, and ½ FSW containing creosote-treated wood. 4A: 5 day old control embryo (1/2 FSW) exhibiting normally pigmented optic vesicles. 4B: 5 day old embryo exposed to untreated-wood with normally pigmented optic vesicles. 4C: 5 day old embryo exposed to creosote-derived compounds. Optic vesicle pigmentation is delayed relative to the controls. 4D: Arrest of development in embryo exposed to creosote-derived compounds. Arrows indicate prominent yolk vacuoles.

Fig. 4

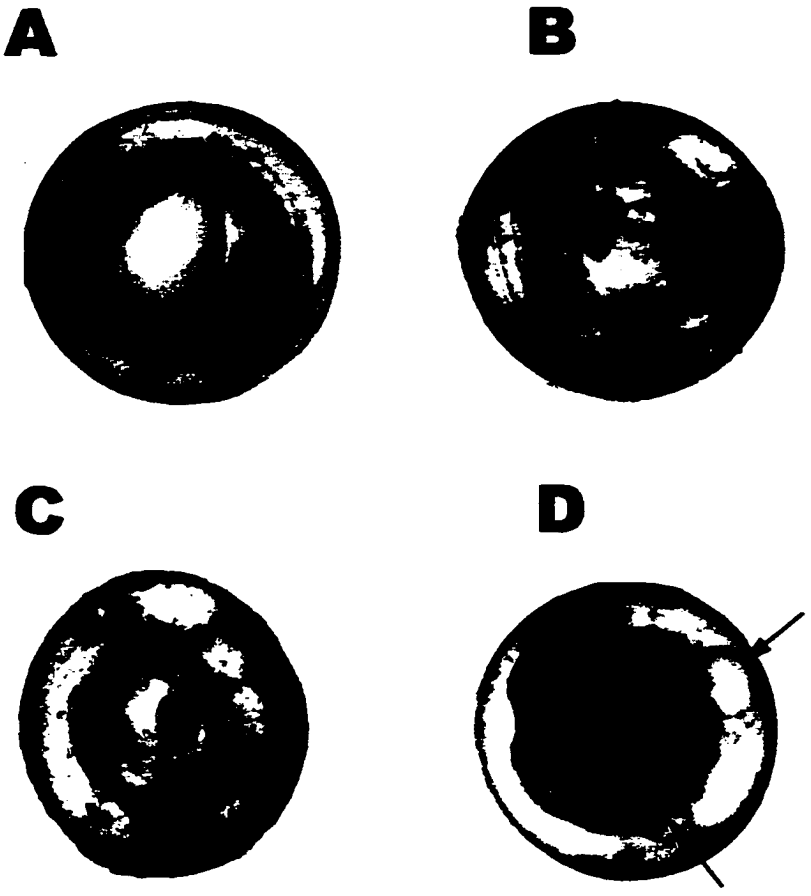


Fig. 5A: Effect of creosote on embryonic heart rate. Embryos were incubated with ½ FSW (control), untreated-wood, or creosote-treated wood. Heart rates in ½ FSW and untreated-wood exposed embryos were not significantly different and tended to increase with incubation age. Heart rates in creosote exposed embryos were significantly lower than either of the controls and decreased over the 5 day observation period. Different letters indicate significant differences with respect to each other at the 0.05 level.

Fig. 5B: Effect of creosote on heart rhythm. Heart contractions of embryos in ½ FSW (upper tracing) were consistently rhythmic. Embryos exposed to creosote-treated wood (lower tracing) exhibited severe arrhythmia as compared to controls in ½ FSW alone. In the graph above, the heart rate of the exposed embryo is fairly rhythmic, although slow, for the 1st 13 seconds, followed by cessation of heartbeats for approximately 6-10 second intervals. At approximately 40 seconds, normal heart rate continues.

Fig. 5a

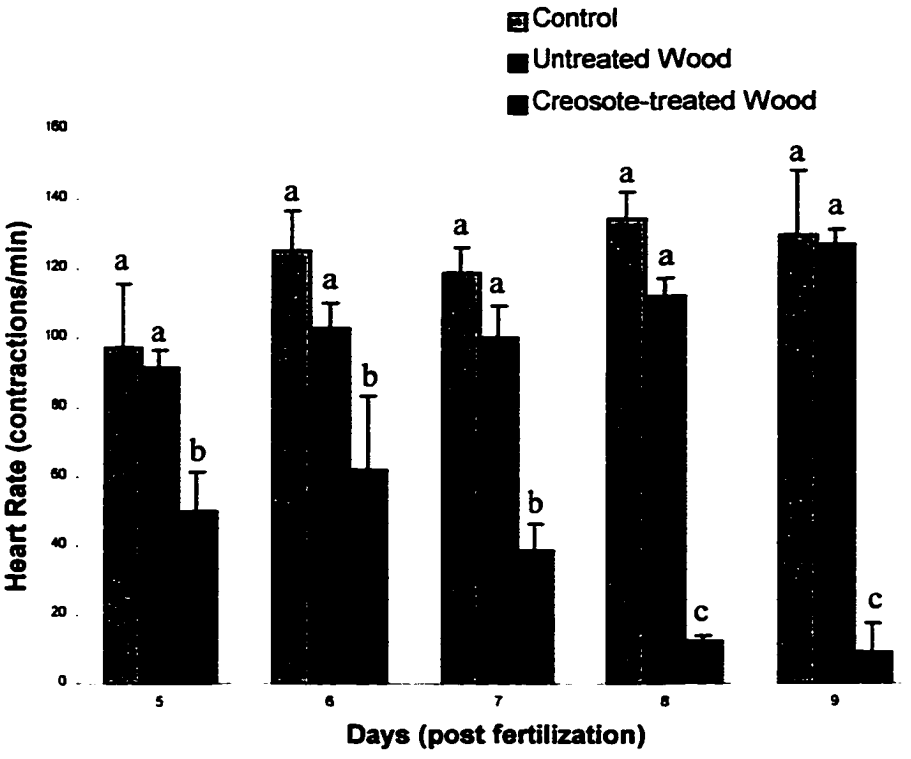


Fig. 5b

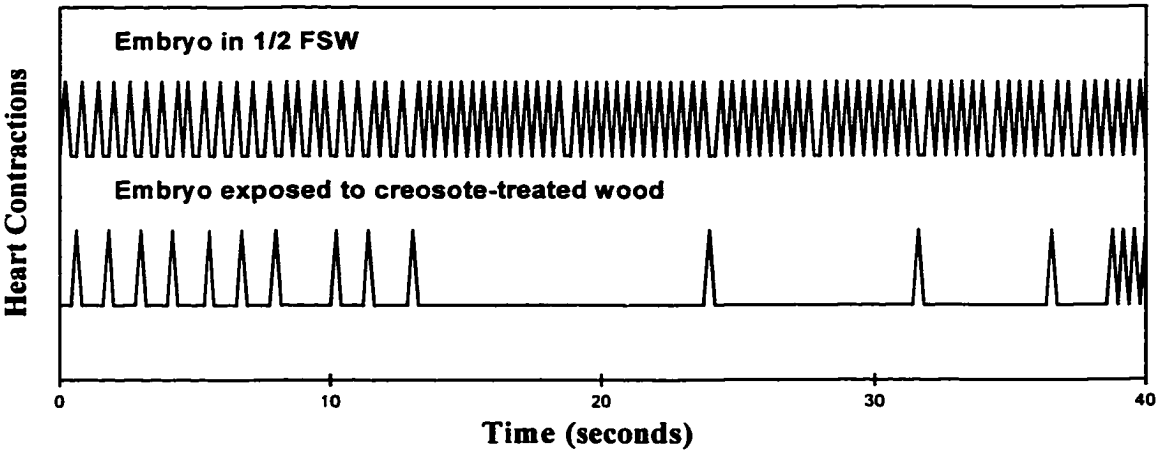


Fig. 6: Effect of creosote on embryonic activity. Control embryos (1/2 FSW and untreated- wood) exhibited vigorous movements within the chorion, decreasing in frequency over the 5-day observation period. Embryonic activity in creosote exposed embryos significantly increased over this same period, but consisted primarily of faint tremors or twitches. a indicates significance at the 0.05 level.

Fig 6

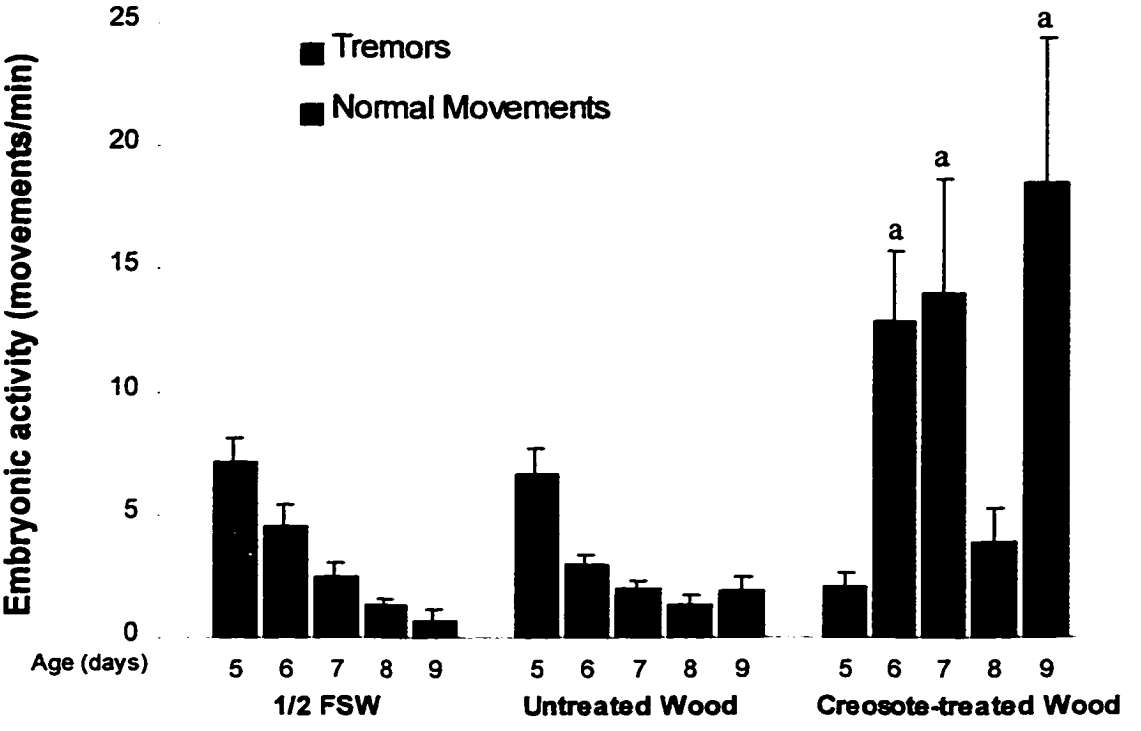


Fig. 7: Effect of creosote on hatching success and normal larval morphology. Hatching rates for ½ FSW controls were typically >80% with most larvae appearing normal. Both creosote-treated and untreated-wood embryos had significantly lower hatching rates. Creosote exposure also resulted in a significant increase in abnormal morphology ($p<0.05$). Different letters indicate significant differences with respect to each other.

Fig. 7

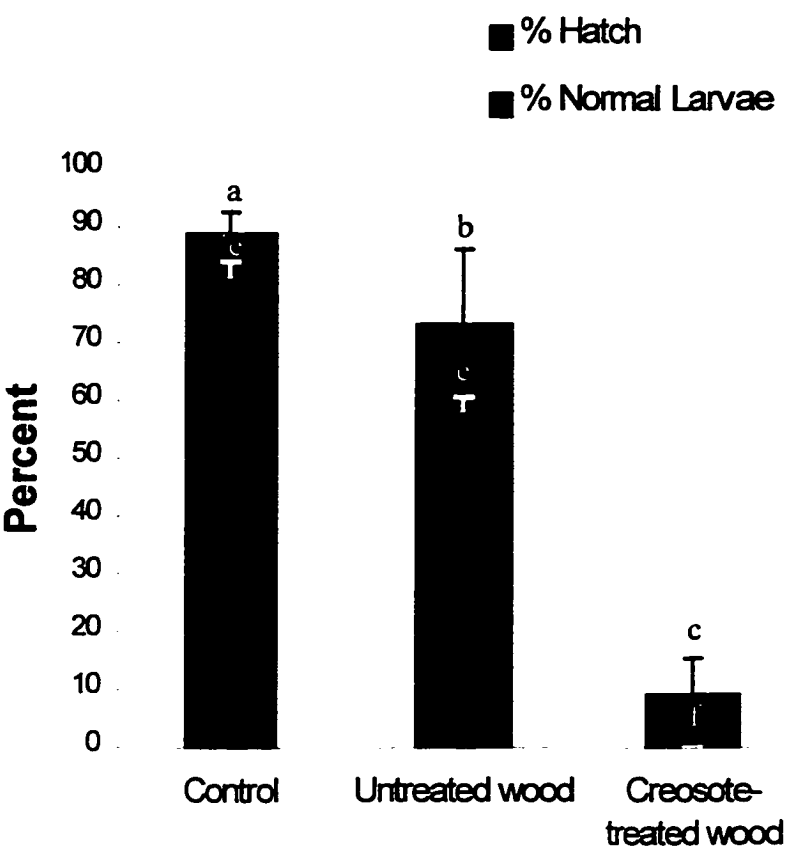


Fig. 8: Effect of creosote-derived compounds on larval morphology. 8a: Control (1/2 FSW) larva exhibiting straight spine. 8b: Larva exposed as an embryo to creosote-derived compounds exhibiting scoliosis of the spine. 8c: Partially hatched dead larva exposed as an embryo to creosote-derived compounds. 8d. Control (1/2 FSW) larva with no evidence of pericardial edema or yolk sac edema. 8e: Larva exposed as an embryo to creosote-derived compounds. Arrows indicate significant pericardial and yolk sac edema. 8f: Larva exposed as an embryo to untreated-wood exhibiting straight spine. Note discoloration of body due to staining by untreated-wood components.

Fig. 8

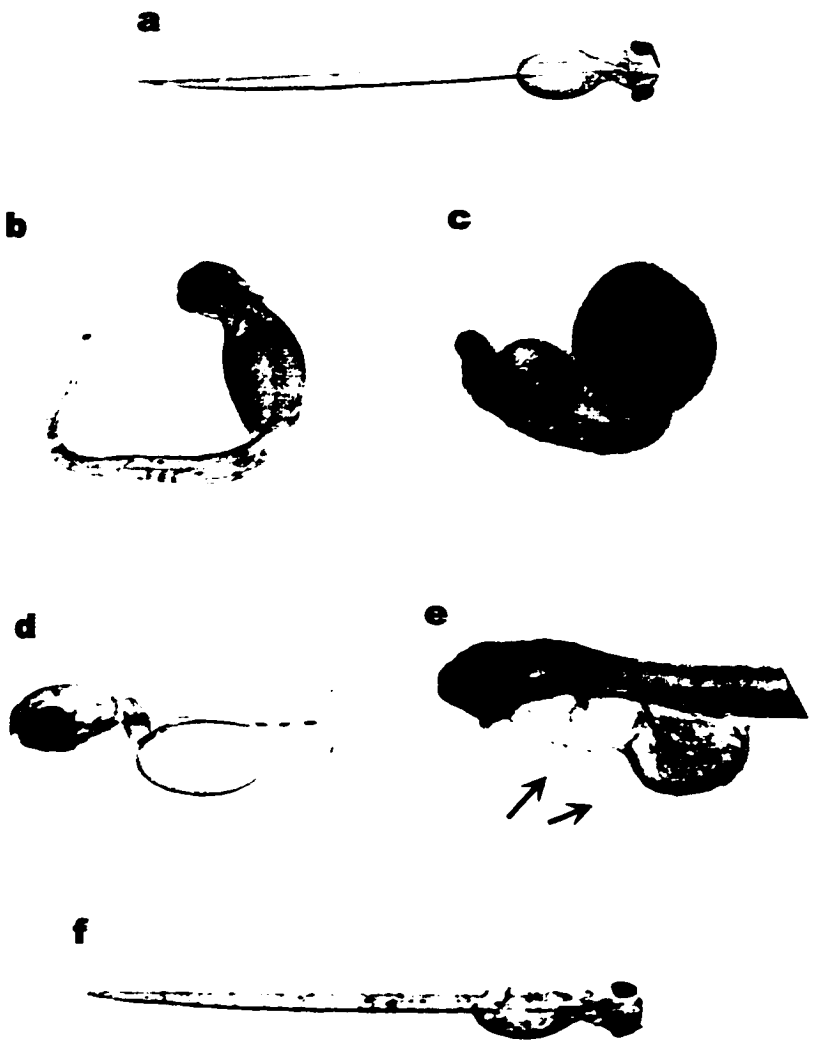


Fig. 9: Combined effects of salinity and creosote. Embryos were incubated in 8, 16 or 28 ppt salinity with or without creosote (3 μg/L). Both hatching and normal morphology were significantly reduced in 8 and 28 ppt salinity as compared to 16 ppt (a denotes a significant difference at the 0.05 level for hatching as compared to 16 ppt; c denotes significance at the 0.05 level for morphology as compared to 16 ppt). The addition of creosote also resulted in significantly reduced hatching rates at all three salinities (b denotes significance in hatching at each salinity). Normal morphology in response to creosote exposure was significantly reduced in 8 and 16 ppt (d denotes significance at the 0.05 level) but not in 28 ppt salinity.

Fig. 9

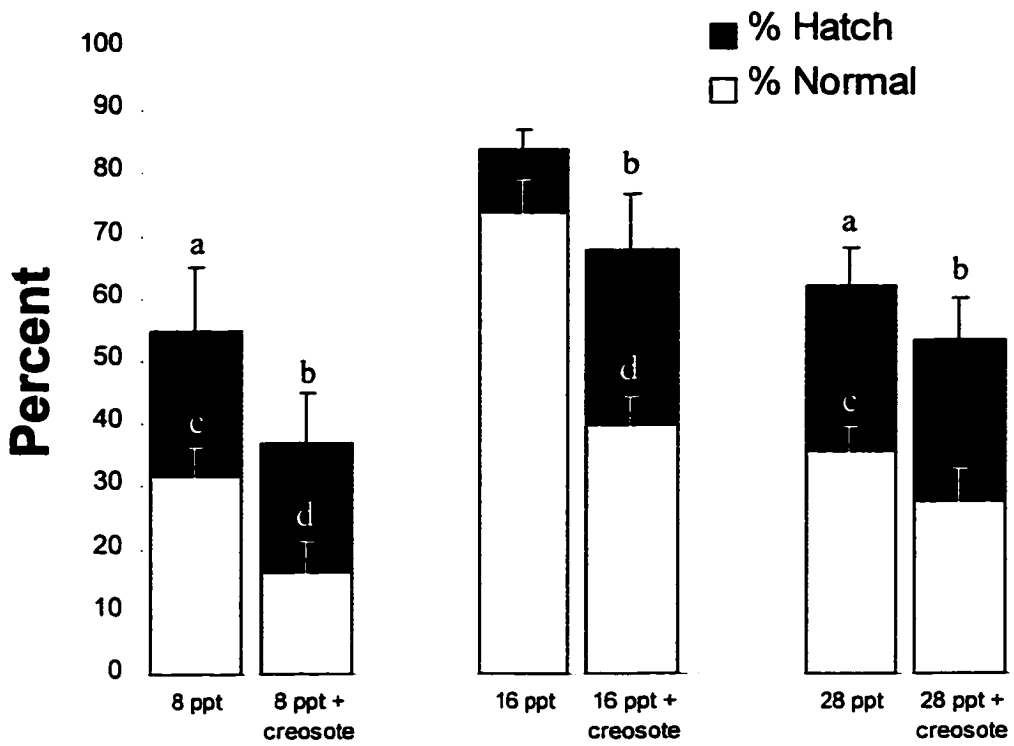


Fig. 10: Effect of salinity and creosote on heart rate. Embryos were incubated in 8,16 or 28 ppt salinities with or without creosote (3 μgl/L). Both 8 and 28 ppt salinities resulted in reduced heart rates as compared to 16 ppt (b denotes significance at the 0.05 level). Exposure to creosote also reduced heart rates in all 3 salinity groups (c indicates significance at the 0.05 level . The additive effect of creosote and salinity was not significant.

Fig. 10

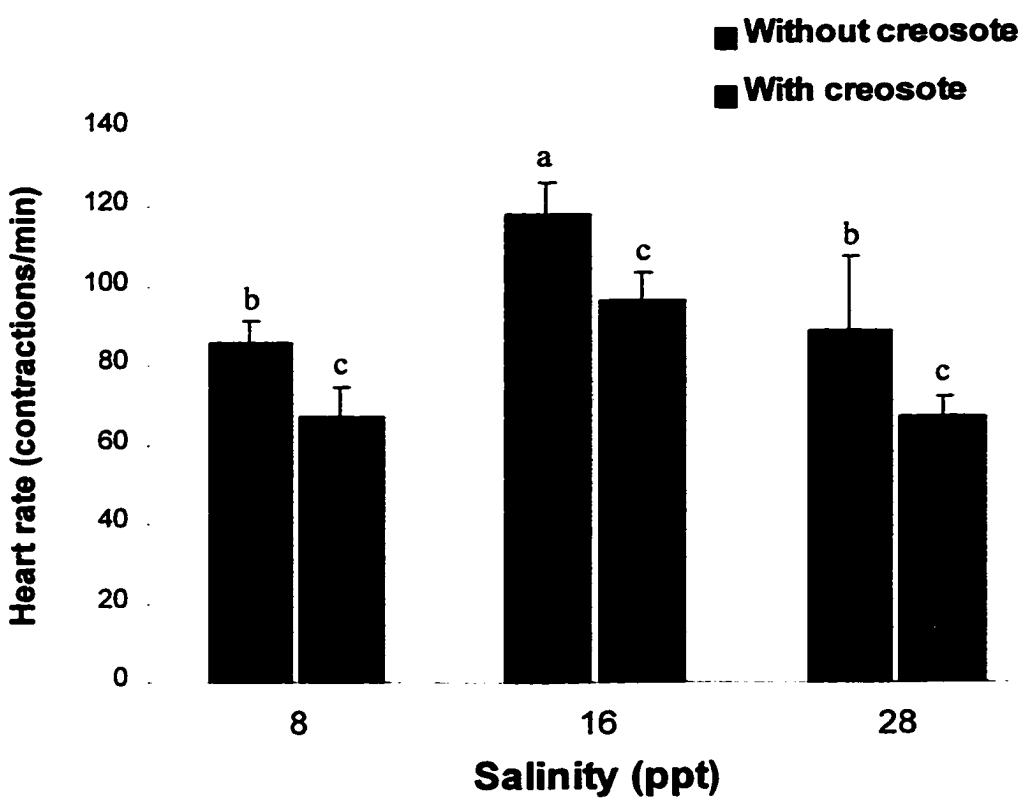
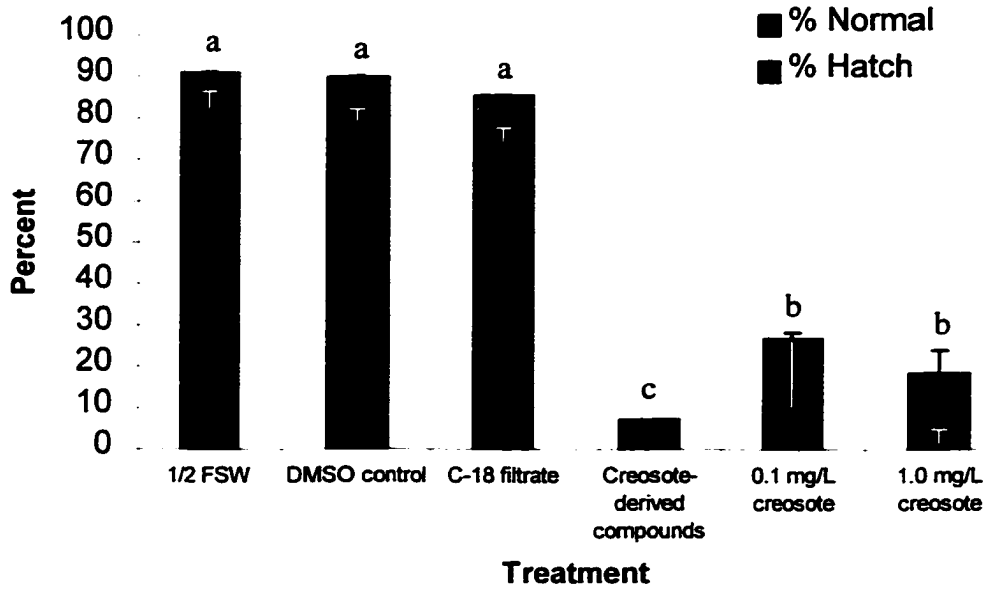


Fig. 11: Effect of commercial coal-tar creosote, and C-18 extracted water on hatching and normal development. Hatching rates and larval morphology were not significantly different between embryos exposed to ½ FSW, DMSO (solvent) control, or ½ FSW containing creosote-derived compounds after C-18 filtration. Hatching rates were significantly reduced in embryos exposed to commercial coal-tar creosote as compared to the controls and C-18 extracted water exposures. Embryos exposed to creosote-derived compounds prior to filtration (1.5 mg/L) exhibited the lowest hatching rates. Different letters represent significant differences with respect to each other.

Fig. 11



CONCLUSIONS

The survival of any species ultimately depends on the ability of that species to successfully reproduce. Early life stages (gametes, embryos, and larvae) are a critical component in this process. For successful fertilization to take place, sperm and eggs must encounter each other and fuse, initiating the program of development leading to an adult organism. For broadcast spawning animals, this entails synchronous spawning of gametes into an aquatic environment which is influenced by a number of biotic and abiotic factors. This dissertation examined the effects of two potential stressors on reproduction in the Pacific herring, a teleost fish that spawns in bays and estuaries (Alderdice and Velsen, 1971; Barnhart, 1988). The first, salinity, can be both a natural stressor (elevated in drought years, reduced in high rainfall (El Nino) years) and an anthropogenic stressor (diversion of freshwater from bays and estuaries). The second, creosote-treated wood in the form of pilings and piers, is an anthropogenic. The effects of these two factors on gamete function (sperm motility), fertilization, or embryonic development were the focus of this study.

In chapter one, salinity tolerance ranges for sperm motility initiation in response to sperm motility initiating factor (SMIF), fertilization, and development (hatching) were determined in Pacific herring from San Francisco Bay. Pacific herring spend most of the year in oceanic waters over the continental shelf, but spawn in lowered salinity waters of the bay during the winter-spring. The salinity history of Pacific herring prior to spawning is not known at this time, however, and they are likely exposed to a wide range of salinities. In comparison, the salinity history of Baltic herring, which reside and spawn in

low salinity waters (4-8 parts per thousand) in Airisto Sound, Finland is well known (Haapala and Alenius, 1994). This study demonstrated that sperm motility, fertilization, and development were all optimal in about 16 parts per thousand (ppt) seawater, approximately on-half the salinity of oceanic seawaters. Embryos that were deployed at two field sites in San Francisco Bay with differing salinity profiles (a "low" salinity site, and a "high" salinity site) exhibited a similar tolerance range for hatching as observed in the laboratory. The salinity tolerance range for the Baltic herring was shifted down to 8 ppt for fertilization and development, which correlates with salinities of their resident and spawning grounds. Sperm motility in Baltic herring was also examined, and found to be similar to that for the Pacific herring, in that motility was initiated in response to SMIF from Pacific herring and in response to an egg-derived protein isolated from Baltic herring eggs. Since elevated salinities inhibit fertilization (Griffin *et al.*, 1998), the effect of major cations found in seawater on fertilization were also examined. Both reduced and elevated sodium (Na^+) inhibited fertilization, and this effect was enhanced if additional potassium (K^+) was added to the incubating media. In general, calcium,(Ca^{2+}) magnesium (Mg^{2+}), or K^+ alone had no effect on fertilization, unless absent from the media. This has previously been shown for Ca^{2+} and K^+ (Yanagimachi and Kanoh, 1953; Yanagimachi, 1956; Yanagimachi *et al.*, 1992).

Herring sperm are unique among teleosts, in that they are immotile at spawning, over a wide range of salinities (Yanagimachi and Kanoh, 1953; Yanagimachi, 1956), but initiate motility in response to SMIF which is localized to the micropyle region of the egg (Griffin *et al.*, 1996). Chapter two focused on changes in intracellular ions associated with initiation of sperm motility in response to SMIF as well as the effect of alteration of

extracellular ions on this process. SMIF induced a depolarization of the membrane, which was inhibited by increasing salinity or extracellular Na^+. SMIF also induced an increase in intracellular Ca^{2+}, which was dependent on the presence of at least 1 mM Ca^{2+} in the extracellular medium. In addition, SMIF induced an efflux of Na^+, which also required the presence of extracellular Ca^{2+}. This data, coupled with the fact that sperm motility can be initiated in the absence of SMIF by using low sodium seawater, suggested that a Na-Ca exchange mechanism working in reverse (calcium in, sodium out) might be involved in motility initiation. Reverse Na-Ca exchange has been demonstrated in a number of tissues, including mammalian smooth muscle, and barnacle muscle (reviewed by Philipson and Nicoll, 1993). However, to our knowledge, this has not been described for any sperm. Membrane depolarization, influx of Ca^{2+}, and Na^+ efflux was inhibited by Na-Ca exchange inhibitors, further supporting this hypothesis. Calcium channel blockers also inhibited these processes, suggesting that calcium channels are likely involved in motility initiation as well. Since males tend to spawn prior to females (Stacey and Hourston, 1982), the presence of a Na-Ca exchange mechanism may be an adaptive strategy to insure that sperm remain immotile until eggs are actually present. Upon contact with the herring egg in the region of the micropyle, sperm motility is initiated. The circular motility pattern observed in response to SMIF suggests that this pattern of motility may be important in maintaining sperm in the micropyle region, since this contains the micropylar canal through which the fertilizing sperm must travel to fertilize the egg. Circular motility has been associated with elevated intracellular Ca^{2+} (Boitano and Omoto, 1991), thus the presence of a Na-Ca exchanger as well as calcium channels may insure that sufficient Ca^{2+} enters the sperm to result in circular motility.

The presence of embryos spawned onto creosote-treated pilings at one of our salinity field sites provided the opportunity to investigate the effects of creosote-derived compounds on development, and this is the focus of chapter three. Creosote is a complex mixture of compounds, including polycyclic aromatic hydrocarbons (PAHs), phenols and creosols, but the majority of toxicity has been attributed to its PAH constituents(U.S. Public Health Service, 1990). PAHs are known to be cytotoxic, genotoxic, mutagenic and carcinogenic (Malins *et al.*, 1985; von Burg amd Stout, 1992; Fournie amd Vogelbein, 1994a,b; Gagne *et al.*, 1995). Embryos exposed in the field had significantly reduced hatching rates and abnormal morphology (scoliosis) as compared to embryos collected a distance from the piling. The study was expanded in the laboratory to examine a number of parameters, including cardiac function, embryonic activity within the chorion, hatching success, and larval morphology. Embryos exposed to creosote-derived compounds were found to have significantly lower heart rates and abnormal heart rhythyms. The pattern of embryonic movement within the chorion was also dramatically altered. In contrast to control embryos which exhibited vigorous twisting movements, activity of exposed embryos consisted of tremors or twitches. As seen in the field, the hatching success of exposed embryos was significantly reduced, and larval morphology was abnormal in all cases (scoliosis, pericardial edema, and/or yolk-sac edema). Dilution of creosote-derived compounds did result in an improvement in these parameters. Finally, exposure to creosote at less than optimal salinities was found to have an enhanced effect on measured parameters. It is estimated that these pilings have been at the field site for about 40 years, a testament to the efficacy of creosote-treated wood in protecting wood products. The overall impact on non-target species, such as herring, is

not known, but should be investigated further to assess the benefits and risks of continued use of this product.

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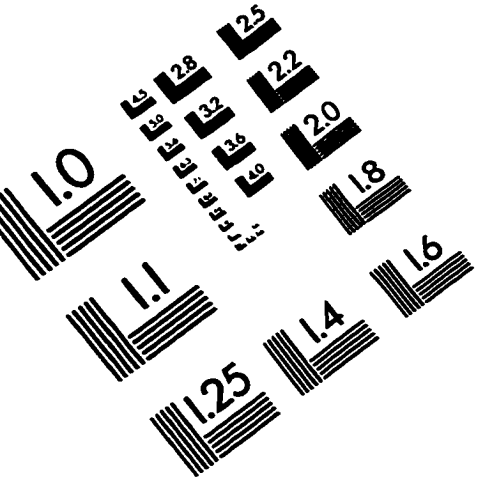
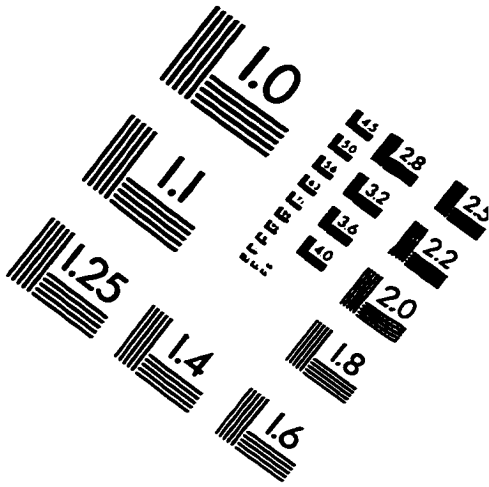
ACKNOWLEDGMENTS

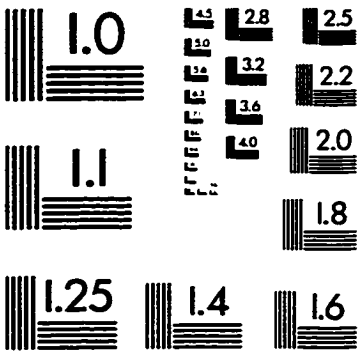
I would like to thank Ken Oda and Diana Watters (California Department of Fish and Game, San Francisco Bay), Tom Moore and John Mello (California Department of Fish and Game, Tomales Bay) Steve Moffitt (Alaska Department of Fish and Game), Dr. Susan Sanders (Bamfield Marine Station), and Juha Kaaria (University of Turku, Finland) for generously providing fish for these studies. This research was funded by California Sea Grant College Program (National Oceanic and Atmospheric Administration, U.S. Department of Commerce, under grant No. NA36RG0537, project No. R/F161) and by the Coastal Toxicology component of the University of Californiai Toxic Substances Research and Teaching Program.

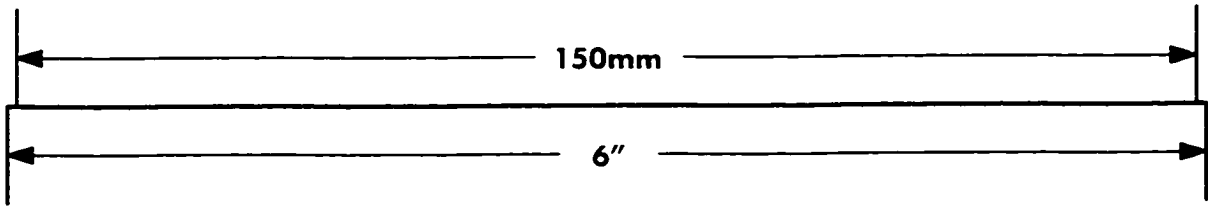
I would like to thank Dr. Gary Cherr for his support, encouragement, ideas, and humor as my major professor. I would also like to thank Dr. David Hinton for his advice and editorial review of this dissertation, and especially for reading it in such a timely manner. I would also like to thank Dr. Frederick Griffin for his advice and keeping me rooted in reality. A special thanks to Dr. Murali Pillai for teaching me laboratory techniques. Thanks also to the following individuals whose help and moral support sustained me throughout this project: Dr. Jill Spangenberg, for moral support and encouragement; Thea-Hibbard Robbins, my invaluable field and lab buddy; Ed Salinas, for helping me with animal care; Dr. Mark Snyder, for nagging me to finish; Dr. Van Peeke, for statistical advice; Dr. Victor Chow, for computer help; Dr. Jim Moore, for computer problems; The laboratory staff of Santa Rosa Memorial Hospital for accomodating my scheduling needs, my friends and neighbors, for feeding me and looking after me; and my family, for encouraging me.

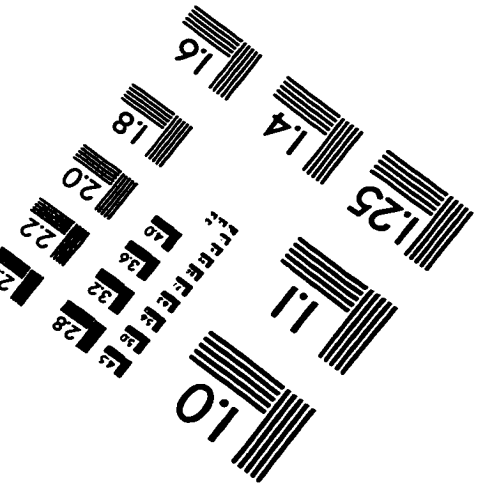
IMAGE EVALUATION
TEST TARGET (QA-3)

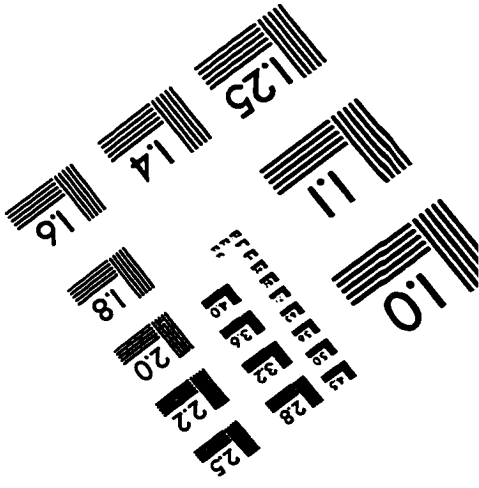












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