PHOSPHORUS COMPOSITION AND FATTY ACID PROFILES AS DETERMINANTS OF EGG QUALITY IN SOUTHERN FLOUNDER
PARALICHTHYS LETHOSTIGMA

Christopher A. Woolridge

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Approved by

Advisory Committee

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Chair

Accepted by

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Dean, Graduate School

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ABSTRACT

We used *in vivo* ^{31}P-NMR to examine biochemical events during development in embryos of southern flounder *Paralichthys lethostigma*. Adult southern flounder broodstock held under an artificial photothermal regime simulating natural seasonal changes spawned volitionally or following hormone induction by LHRH-a. Buoyant eggs were collected and incubated in 34-ppt seawater at 16º C, and their fertilization rate was monitored. Eggs of a prescribed developmental stage were transferred from the incubator to aerated seawater in a 5-mm NMR tube, and spectra were collected immediately. ^{31}P-NMR peaks were observed for inorganic phosphate (Pi), the α, β, and γ-phosphates of ATP, NAD(P)H, phosphocreatine (PCr), sugar phosphates, and the phospholipids: phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylethanolamine (PE). Levels of PC, PI, and α, β, and γ-phosphates of ATP, remained at relatively constant levels in unfertilized eggs over time. However, in eggs that were fertilized, there was a significant increase in PE as they developed, which was not observed in unfertilized eggs. Using the chemical shift of the Pi peak, intracellular pH (pHi) was determined. In fertilized floating eggs the pHi decreased from 7.1 (15 h post-spawn) to 6.2 (24 h post-spawn) and then increased to 6.8 (48 h-post-spawn). ATP showed an inverse relationship with pH, starting to decline when pH reached its minimum value. This suggested that ATP was used to stabilize embryonic pH through active transport of nitrogenous waste, such as ammonia, outside of the developing embryo. By the pre-hatching embryo stage, the ATP phosphates, PCr, and NAD(P)H

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levels declined to undetectable levels, indicating an embryonic requirement for these compounds.

Fatty acid profiles during development in embryos were also examined using HPLC. Lipid and fatty acid composition were compared between embryos (blastula stage) of high quality (fertilization rate = 91.5%, survival to first feeding = 39.0%) and low quality (fertilization rate = 39.0%, survival to first feeding = 13.1%). The most abundant fatty acids (proportion of total fatty acids) included, 16:0 (palmitic acid, 21.5%), 18:1 n-9 (oleic acid, 17.5%), and 22:6 n-3 (docosahexanoic acid, DHA, 26.1%). There were no significant differences ($P > 0.05$) between low and high quality embryos in total lipids (4.4% wet wt), DHA (26.7%), EPA (3.3%) and ARA (1.9%) and DHA/EPA ratio (8.3). There were no significant differences ($P > 0.05$) in total lipid and fatty acid profiles in high quality eggs at the blastula, gastrula, early embryo, and late embryo stages.

In summary, southern flounder eggs that were viable were characterized by an increase in PE during development and decreasing levels of ATP, PCr, and NADP(H). The increase in PE is possibly a way to conserve DHA important to neuro-visual tissue development. Southern flounder eggs contained high levels of polyunsaturated fatty acids, mainly DHA. High levels of the saturate, palmitic acid, as well as the monounsaturate, oleic acid were also present. Fatty acid profiles were not correlated with egg quality. Lack of significant decreases in phospholipids and fatty acids during embryogenesis suggested that other components (e.g. glycogen and free amino acids) accounted for embryonic energy needs. We hypothesize that in southern flounder, lipid is conserved for utilization after hatching, and that catabolic substrate consumption in

embryos starts with glycogen, followed by free amino acids, and then lipid after hatching.

We further hypothesize that this pattern may be characteristic of subtemperate marine

fish eggs with an oil globule.

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INTRODUCTION

In recent years, there has been an increase in interest in production of North American flatfishes for sale as a mariculture product and for use in possible stock enhancement programs (Copeland et al., 1996). The stimulus has been the reported successful culture of various flatfish species including Japanese flounder, *Paralichthys olivaceus* (Tsujigado et al., 1989) in Japan and Korea, and turbot, *Scopthalmus maximus* (Bromley et al., 1986; Devauchelle et al., 1988) sole, *Solea solea* (Devauchelle et al., 1987) and Atlantic halibut, *Hippoglossus hippoglossus* (Holmefjord et al., 1993) in Northern Europe.

Southern Flounder

Southern flounder, *Paralichthys lethostigma,* is the largest flounder of the family Bothidae occurring in the inshore waters of the South Atlantic and Gulf coasts of the U.S., reaching up to 9 kg. It is an important recreational and commercial species (Wenner et al., 1990) and is the number one flatfish species landed in NC. In 1997, 7.9 million kg were landed in NC worth an estimated $1.8 million (Safrit and Schwartz, 1998). According to the NC Division of Marine Fisheries, the population biomass of southern flounder in NC declined by 32% from 1993-2002, due in part to over-fishing.

From spring through fall, southern flounder inhabit coastal bays, sounds, and lagoon and river systems, occasionally entering fresh water (Ginsburg, 1952; Gutherz, 1967; Dahlberg, 1972). Although they may reside in estuaries throughout the year (Dahlberg, 1975), adults migrate offshore to spawn in late fall and winter (Ginsberg,

1952; Stokes, 1977). Southern flounder have been shown to grow well in fresh and salt water (Lasswell et al., 1977; Daniels et al., 1996). This euryhaline ability has generated considerable interest in their aquaculture potential.

Southern flounder is a prime candidate for commercial aquaculture due to their wide salinity tolerance and strong international market. Several studies have been done to develop captive spawning techniques (Arnold et al., 1977; Henderson et al., 1988; Berlinsky et al., 1996; Watanabe et al., 2001) and larviculture methods (Daniels et al., 1996; Denson and Smith, 1997; Jenkins and Smith, 1997; Smith et al., 1999; Daniels and Watanabe, 2003) for the southern flounder. A bottleneck still exists at the larviculture stage for southern flounder, as too few fingerlings are being produced to encourage the development of grow-out facilities (Van Maaren and Daniels, 2000). Southern flounder may become one of the first marine species extensively farmed in the United States; however, large-scale production of flounder larvae is currently hampered by inconsistent egg quality in wild and captive broodstocks (Copeland et al., 1996).

Egg Quality

One of the most important constraints to the growth of the aquaculture industry is the variable quality of the eggs that are available for growing market-sized fish (Bromage et al., 1994). Varying egg quality has been identified as a primary factor limiting successful mass production of marine fish larvae (Kjorsvik et al., 1990). The current worldwide growth of aquaculture has created a demand for a reliable source of healthy juveniles over a wide range of fish species. The importance of studies on determining egg quality in marine teleosts has become more acute since more and more new marine

species are being considered for commercial culture (Al-Marzouk et al., 1995). Knowledge about marine finfish egg quality and composition has recently become more available for a wide range of species. New studies of some important cultured marine species and their egg quality include Asian seabass, *Lates calcarifer* (Nocillado et al., 2000), common wolffish, *Anarichas lupus* (Tveiten et al., 2001), Japanese flounder, *Paralichthys olivaceus* (Furuita et al., 2003) and chinook salmon, *Oncorhynchus tshawytscha* (Wendling et al., 2000).

In the literature, attempts have been made to define egg quality in various ways by different authors. However, the definition that has general validity is "the egg's potential to produce viable fry" (Kjorsvik et al., 1990). A wide array of physical and biological factors can affect egg quality (Bromage et al., 1994). Good eggs should have high fertilization rates and hatching rates as well as correspondingly high survival rates through the transition phase from endogenous to exogenous nutrition (Al-Marzouk et al., 1995). The stage that marks the onset of exogenous feeding is termed first-feeding. First-feeding larvae are first fed live feeds and then weaned to artificial diets. When larval condition at first-feeding is optimized, then high survival and good growth to more advanced stages can be achieved. In order to facilitate juvenile production, a source of high quality eggs is required along with the ability to identify what batches of eggs are of sufficient quality to promote good larval survival and growth. A hatchery manager can not afford to waste precious resources in the form of time, personnel, and feed on eggs and larvae of poor quality.

Egg Composition

It has been suggested that the specific endogenous fuels consumed during the yolksac stage reflect the nutritional requirements of the larvae when converting to exogenous feeding (Hemming and Buddington, 1988; Fyhn, 1989). Neutral lipid or triacylglycerol is generally considered to be the most important energy reserve in marine fish eggs (Vetter et al., 1983; Blaxter, 1969) although both protein (Lapin and Matsuk, 1979; Buckley, 1981; Cetta and Capuzzo, 1982) and carbohydrates (Hemming and Buddington, 1988) have been shown to be important in some species. Free amino acids (FAA) have also been implicated as a fuel in energy metabolism of marine eggs and larvae (Fyhn and Serigstad, 1987; Fyhn, 1989, 1990; Ronnestad, 1990; Ronnestad and Fyhn, 1992).

Eggs derive their endogenous resources from their maternal adults. Thus, if there is an irregularity in some aspect of broodstock nutrition, this can affect egg quality and larval survival. This can be avoided by feeding broodfish well-balanced diets with essential components such as protein and essential fatty acids (EFA), derived from fish meal. These diets are occasionally fortified with vitamins and minerals. In a review of broodstock nutrition and reproductive performance, Izquierdo (2001) suggested that lipid and fatty acid composition of broodstock diets directly influence reproductive success and offspring survival. The relationships between egg fatty acid and phosphorus compound composition and egg quality are not well understood in marine finfish, including the southern flounder.

Lipids

Lipids and their constituent fatty acids are important for meeting fish embryonic and larval energy requirements (Sargent, 1995a), supplying structural components of membranes, as well as functioning in cell regulation (Tocher et al., 1985; Falk-Peterson et al., 1986; Fraser et al., 1988; Evans et al., 1996). In turbot, *Scopthalmus maximus*, lipids derived from the oil globule seem to be the main fuel after hatching and account for approximately 90% of the energy dissipation at the onset of first-feeding (Ronnestad et al., 1992). Differences between the fatty acid profiles of eggs from wild versus cultured fish and repeat spawners versus first-time spawners have previously been used as evidence of a biochemical basis for variable egg quality (Evans et al., 1996).

Fish naturally contain relatively high levels of the highly unsaturated fatty acids (HUFA), docosahexaenoic acid (DHA), 22:6(n-3), and eicosapentaenoic acid (EPA), 20:5(n-3), in their body tissues and consequently have high nutritional requirements for these fatty acids. The n-3 polyunsaturated fatty acid (PUFA) content of eggs from wild European seabass *Dicentrarcus labrax,* gilthead seabream *Sparus aurata*, and red seabream *Pagrus major* contained higher amounts of DHA and EPA than eggs from cultured counterparts (Komis et al., 1990). It has been shown in Atlantic halibut that larger repeat spawners allocated more lipid material (total lipid, triacylglycerol, sterol, DHA and arachidonic acid (AA) to eggs and had higher fertilization rates than first-time spawners (Evans et al., 1996). This suggests that smaller, first-time spawning, broodstock could be deficient in important nutrients required for good egg quality.

Marine finfish larvae experience an exponential growth phase that is more energy dependent than older juveniles and probably have a higher lipid requirement. Marine

finfish generally cannot convert 18:3(n-3) to 20:5(n-3) due to low Δ-5 fatty acid

desaturase activity. There is considerable evidence (Sargent et al., 1993, 1995a) that

those marine fish studied so far can convert 20:5(n-3) to 22:6(n-3), but at low rates not

likely to meet fully the high demand for 22:6(n-3) during larval growth (Sargent et al.,

1997). Watanabe and Kiron (1985) have revealed the importance of pre-spawning

nutritional regimes and more specifically the levels of (n-3) PUFA (i.e. DHA and EPA)

in the diets of broodfish on egg and larval quality. In this regard, not only can the

absolute levels of EPA and DHA, but also the ratios of DHA: EPA and (n-3): (n-6)

PUFA in the marine teleost broodstock diet, be considered as important determinants of

egg quality. Since eggs derive their resources from female broodstock, DHA and EPA

can be considered essential dietary requirements not only in larvae, but in southern

flounder broodstock as well. By studying biochemical changes that occur during

embryonic and early larval development, probable nutritional requirements of first-

feeding larvae can be estimated (Fraser et al., 1988). The DHA and EPA content in

marine finfish embryos may help determine the "optimal" larval dietary DHA and EPA

requirements at first-feeding associated with the highest survival and growth (i.e.

"quality"). Recently, arachidonic acid (AA), 20:4 (n-6), has been shown to be an

important component of Japanese flounder broodstock diet for high larval quality, with a

supplement of AA at 0.6 g / 100 g diet showing the best results (Furuita et al., 2003).

According to a review by Rainuzzo et al., (1997) good quality larvae should have

enough energy stores in the form of phospholipids (PL), in particular phosphatidylcholine

(PC), to sustain the transition from endogenous metabolism to exogenous feeding. This

was demonstrated for plaice, halibut and cod. Prior to exogenous feeding,

phosphatidylethanolamine (PE) tended to be produced in all four species (Rainuzzo et al., 1997). It has been acknowledged that PC is an important source of essential fatty acids (EFA), organic phosphorus, and choline in eggs and larvae of cod (Fraser et al., 1988). The consumption of PC was related to a reduction in DHA in these species. The biosynthesis of PE has been observed concurrently with PC catabolism in halibut, plaice and cod (Fraser et al., 1988; Rainuzzo et al., 1992).

Phosphorus

Inorganic phosphorus has been determined to be important in marine finfish development. An important component of ATP, inorganic phosphorus is necessary for metabolic energy. For example, in red sea bream, *Pagrus major*, eggs low in phosphorus were of low quality and low phosphate diets fed to broodstock prior to spawning deleteriously affected the subsequent egg quality (Watanabe, 1982).

Carotenoids

Egg pigment content is another important component of fish eggs. Carotenoid content of salmonid eggs has been strongly correlated with good egg quality (Craik, 1985). Carotenoids have antioxidant properties and are capable of binding to free radicals.

Morphology

Symmetrical blastomere morphology, or symmetry of egg cell division, has been demonstrated to be positively correlated with halibut egg viability, and observations of egg blastomere morphology can provide commercial hatcheries with a rapid method for checking quality (Shields et al., 1997).

Hormone induced spawning

Hormonal treatments to induce spawning in teleost fish are used for a number of cultured fish species (Crim et al., 1987). Advantages to manipulation of spawning through hormone introduction include the selection of superior individuals for breeding to improve culture performance (e.g. faster growth) and using these maturation hormones is convenient by timing strip spawns according to the hatchery manager's schedule, which is important due to manpower requirements and high cost of growing larval live diets. For some species, hormone induced spawning may be the only means of producing fertilized eggs from captive broodstock (Ako et al., 1994). Successful induction of spawning of captive winter flounder, *Pseudopleuronectes americanus*, was reported using human chorionic gonadotropin (HCG), carp pituitary extract (CPE) (Smigielski, 1975), and a synthetic mammalian gonadotropin releasing hormone analog (GnRHa) known as leuteinizing hormone releasing hormone analogue (LHRHa) (Crim, 1985; Weigand et al., 1987).

Results of previous studies of egg quality and larval development following hormonal induction of spawning have been mixed. Good egg quality in terms of high hatching rates and subsequent larval survival was reported following LHRHa-induced spawning in the winter flounder (Harmin and Crim, 1992). In contrast, poor egg quality

was observed in terms of fertilization success and egg viability following injection of LHRHa in the California halibut, *Paralichthys californicus* (Caddell et al., 1990). On the other hand, high hatching rates and a high yield of normal fry were observed in the African catfish, *Clarias gariepinus*, (De Leeuw et al., 1985; Manickam and Joy, 1989) and the Chinese carp (Peter et al., 1988) following GnRHa treatment. In brown trout, *Salmo trutta*, it was hypothesized that GnRHa-induced maturation caused a reduction in egg quality by disrupting the timing of final oocyte maturation and ovulation (Mylonas et al., 1992). Southern flounder have been successfully spawned with LHRH-a cholesterol-cellulose implants after ovarian maturity was achieved by photothermal conditioning of broodstock; however, in vitro fertilization and hatch rates were highly variable and often decreased with successive spawns (Berlinsky et al., 1996), suggesting that essential nutrients necessary for good egg quality were severely depleted in broodstock by repeated spawning. Hormonal treatment, either through injection or implantation of a sustained release LHRH-a pellet, involves handling of broodstock fish, which can be stressful.

More recently, Watanabe et al. (2001) demonstrated that southern flounder broodstock that were photothermally conditioned to ovarian maturity, which involves adjusting the light and temperature cycle to simulate seasonal changes, spawned naturally, producing eggs of higher quality versus eggs produced through LHRHa implantation and hand-stripping. It has been demonstrated in milkfish, *Chanos chanos*, that although LHRHa implanted individuals produced significantly more eggs, naturally spawned eggs were significantly larger in mean egg diameter and showed higher fertilization rates per spawning event (Ako and Lee, 1994). While no significant

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differences were observed for amounts of 18:1(n-9), 18:3(n-3), 22:1(n-11), and 22:6(n-3), all other essential fatty acids as well as the majority of essential amino acids and total protein were significantly higher in naturally spawned eggs (Ako and Lee, 1994). It was suggested that a lower level of arachidonic acid (i.e., 20:4(n-6)) found in eggs produced through hormone induced spawning may contribute to the lower fertilization rates of those eggs. These workers recommended that, since high quality eggs (as measured by fertility rate) can be produced without hormone treatment, hormone-induced spawning of milkfish should be performed only for specific purposes such as selective breeding and/or out-of-season egg production (Ako and Lee, 1994).

Potential Contributions of Study

More information on the biochemical composition of eggs of differing quality and on the effects of different methods of broodstock husbandry (e.g. hormonal implants) on egg biochemistry is needed to understand egg quality and the factors that influence it. In order to elucidate the problem of improved survival of some batches of eggs over others, more information is needed on the specific components of these eggs. Understanding the phosphorus compound content and fatty acid profiles of good batches of southern flounder eggs is a beginning of an investigative process which may one day make it easier to distinguish good and undesirable eggs. With such information, we may be able to enhance survival of these eggs through proper control of husbandry practices including environmental parameters, nutrition and methods for spawning.

Objectives:

I.) To determine the phosphorus and fatty acid profiles of eggs naturally tank spawned from wild-caught captive southern flounder broodstock under photothermally-induced gonadal conditioning.

A) Does egg composition vary for eggs of different quality measured by fertilization and hatching success and survival to first-feeding, and is there an "optimal" phosphorus and/or fatty acid composition correlated with higher quality eggs?

Null: The egg phosphorus and/or fatty acid composition does not differ for eggs that vary in quality.

II.) To determine the changes in phosphorus and fatty acid profiles during the course of embryonic development to hatching.

A) How does the egg composition change during embryonic development for naturally-spawned eggs and for eggs originating from hormonally-induced spawning of similar quality?

Null: There are no significant differences in egg composition over the course of development through first feeding for naturally-spawned and hormonally-induced eggs.

METHODS

This study was conducted at the University of North Carolina at Wilmington Center for Marine Science, (UNCW-CMS) Aquaculture Facility in Wrightsville Beach, NC, between December 2000 and May 2003.

Experimental Animals

Commercial fishermen using pound nets in the Pamlico Sound estuary of NC collected adult broodstock southern flounder. Fish were conditioned in 9.14 m and 18.29 m diameter concrete circular tanks at a grow-out facility in Cedar Island, NC. Broodstock were fed a variety of cut fish and live fish such as striped mullet for a period of a few months up to a year. Broodstock were hand selected based on size for females and spermiation for males. Flounder were transported to the UNCW-CMS Aquaculture Facility where they were stocked into a controlled environment experimental broodfish system.

Experimental System

The controlled environment broodfish tank system consisted of six outdoor, circular, fiberglass tanks (diameter = 2.46 m; depth = 1 m; volume = 4.76 m³). Tanks were insulated and fitted with a conical fiberglass cover, each containing a timer-controlled, fluorescent fixture, containing two 20-watt daylight bulbs providing an average light intensity at the water surface of 234 lux. These tanks were painted black on the inside and external light was kept to a minimum. Tanks were divided into three groups of two, each group supported by a common recirculating system, consisting of a

high-rate sand-filter, fluidized bed filter, foam fractionator and ultraviolet sterilizer. Flounder were weighed, measured and evenly distributed into each tank. Each tank was stocked with approximately 15 adult fish with a male to female ratio of approximately 1:3. Water in each system was exchanged at a rate of approximately 10% per day. Water from each tank drained through an egg collector (diameter = 0.76 m; depth = 0.76 m; volume = 0.24 m³) before entering a reservoir tank (diameter = 1.54 m; depth = 1 m; volume = 1.86 m³), from which water was pumped to the biofilter system. Temperature, salinity, dissolved oxygen, and pH were monitored daily, while total ammonia-nitrogen, nitrite and nitrate were monitored weekly. Temperature was controlled using a heat pump (3 hp) and kept at approximately 16°C (+/- 1°C) during spawning season, from late December to late April. Each tank was placed on a 14 hour dark: 10 hour artificial light cycle throughout the spawning season (Dec.-May) (Watanabe et al., 2001). During the non-breeding season, late spring through fall (May-early Dec.), each tank was placed on an ambient photo-thermal regime. Temperatures were held at a maximum of 26°C, while maximum photoperiod was 14 hours light. Fish were fed daily to satiation thawed Atlantic silversides *Menidia menidia*, supplemented with multi-vitamin and vitamin C tablets.

Experimental Design

The experimental design consisted of two parts: In the first part, biochemical composition of high and low quality eggs produced by natural and hormone induced methods were compared. In the second part, egg biochemical profiles were compared at

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different stages of development to determine embryonic requirements for fatty acids and phosphorus compounds.

Part 1. Comparison of different quality eggs produced by natural and hormone-induced spawning

To compare the biochemical composition of different quality eggs produced by hormone-induced and natural spawning, eggs of similar developmental stage and fertilization rates were analyzed for phosphorus and fatty acid composition. Over the course of the spawning season, eggs obtained from natural and hormone-induced spawning were incubated under controlled conditions and hatching rates and survival to the first feeding stages were monitored as indices of quality. Three categories of egg quality were used as follows: low quality, defined by less than 25% survival to first feeding, and high quality, defined by greater than 45% survival to first feeding and unfertilized eggs. Phosphorus composition was determined for high and low quality eggs across 5 stages of development as follows: unfertilized (0-4 hours post-spawn), blastula (12-16 hours post-fertilization), gastrula (20-24 hours post-fertilization), early embryo (35-40 hours post-fertilization), and late embryo (65-72 hours post-fertilization). Fatty acid composition was determined for eggs in each of these three categories of quality at the blastula stage of development.

Part 2. Egg biochemical profiles during development

To determine changes in biochemical profiles of eggs during development, eggs obtained from natural and hormone-induced spawns were incubated under controlled conditions and hatching and survival rates were monitored as indices of quality. A sample of eggs was also incubated separately and sampled at the following stages of

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development: blastula, gastrula, early-embryo, and late-embryo. Phosphorus and fatty

acid composition were determined for eggs at these stages of development. Only high

quality embryos (>80% fertilization) were used for these determinations.

Hormone-induced Spawning

Mammalian leutenizing hormone releasing hormone analog (LHRH-a) was used

to induce spawning. LHRH-a [D-Ala^6 Des-Gly^{10}]-LH-RH Ethylamide (Sigma Chemical

Co., Missouri, USA) was formed into pellets containing 95% cholesterol, 5% cellulose

(Sherwood et al., 1988). Females with mean vitellogenic oocyte diameters (MOD)

ranging from 411- 523 μm (Shehedeh et al., 1973) were anesthetized and implanted

intramuscularly with a single pellet at a nominal dose rate of 50 μg/kg body weight

(range = 47.2-55.7 μg/kg body weight). Females were treated individually and returned

to the broodtank at a temperature range of 16-18°C. Using a "light" table consisting of a

clear plexiglass table lit from underneath by a 1,000-W bulb, female ovarian development

was monitored in silhouette. Anesthetized females were rated from 0-4, depending on

stage of ovarian development: 0, no ovarian development; 1, ovaries were slightly

darkened, but do not extend back; 2, ovaries were darkened and extended all the way to

the caudal fin, but the fish was not swollen near the oviduct; 3, ovaries were the darkest

extended all the way back and were swollen towards the oviduct; and 4, ovaries were

similar to stage 3, but with a clear spot approximately 4 cm in diameter near the oviduct

(indicating the presence of hydrated ovulated eggs).

To strip spawn, milt was first collected from 1-2 anesthetized (2.5 ppm 2-

phenoxyethanol) males. The area surrounding the genital pore was rinsed with fresh

water (0 ppt) and blotted dry with a paper towel to prevent sperm activation and contamination. Milt was obtained using gentle pressure on the abdomen and collected with a Pasteur pipette as it was expressed from the genital pore. Collected milt was placed into a clean, plastic test-tube on ice. Once at least 1 mL of milt was collected, it was observed for motility using a compound microscope. A small drop was placed on a glass slide, and then a small amount of sea water was added to activate the sperm. After approximately 10 seconds, sperm motility was indexed from 0-4, with 0 meaning no motility and 4 indicating maximum motility. At least 1 mL of motile sperm was collected (index 3-4) for strip spawning trials.

After sperm was collected, eggs were stripped from the anesthetized female. Females were strip-spawned approximately 48-hours after LHRH-a implantation. To determine suitability for strip-spawning, the female was examined on a light table to observe stage of ovarian development. Only females with an index of 4 were selected for hand stripping. The anesthetized female was rinsed with fresh water and blotted dry using paper towels around the abdomen and genital pore. Eggs were stripped by abdominal massage into a 600-mL beaker and care was taken to prevent contamination by water or urine.

Milt was added to the eggs and gently swirled. Once eggs and milt appeared homogenous, 150 mL of filtered seawater at 16-17˚C was added. The eggs were swirled for 3 minutes, and then another 150 mL of seawater was added. Eggs were transferred to a 1,000-mL separatory funnel to separate buoyant (viable) from non-buoyant (non-viable) eggs and the number in each fraction was estimated using volumetric methods (1,000 eggs/mL). Approximately 5,000 floating eggs were placed into 15-L incubators supplied

with flow-through seawater at 35 ppt and aeration. Fertilization success was determined after 2-3 hours by taking a small sample (100 eggs) and counting fertilized eggs out of the total number of eggs. Hatch rates and survival rates were monitored through first-feeding stage (approximately 3 and 7 days post-fertilization, respectfully). Sampling was performed using a vertical volume sample tube and counting larvae on a dissecting scope.

Natural Spawning

Southern flounder often spawned volitionally (without hormone intervention) in the brood tank (Watanabe et al., 2001). Spawned eggs were siphoned from the egg collector into a 250-μm mesh bag, then transferred into a 15-L separatory funnel in seawater where buoyant and non-buoyant eggs were separated and quantified using volumetric methods. Fertilization, hatching, and survival to first-feeding were monitored as described above.

Egg Biochemical Composition

Part 1. NMR analysis

Eggs were sampled for phosphorus composition and fatty acid analysis. Approximately 1,000-2,000 eggs were sampled for phosphorus composition and were transferred immediately in aerated seawater for NMR analysis. Developing eggs were immediately transferred from aerated seawater at a prescribed stage of development into a 5-mm NMR tube. Excess water was strained out by using a metal sieve, and a plastic transfer pipette was used to transfer the eggs into the NMR tube. The NMR tube was then placed inside a Bruker spectrophotometer where phosphorus spectra were recorded

at 400 MHz. It has been shown that ^{31}P-NMR can be successfully applied to a developing biological system such as fish eggs, without affecting its normal capacity to develop (Wasser et al., 1996; Grasdalen and Jørhensen, 1985). A chromatograph was printed out for each sample and peaks were identified using purified phosphorus compounds as standards. Individual peak area was directly correlated with relative abundance, which was converted to a percent. This was accomplished by adding all peak areas and dividing an individual peak area by the total area.

Part 2. Fatty acid analysis

Eggs sub-sampled for fatty acid analysis were placed on a 75-μm mesh Nitex ® screen and blot dried using a paper towel and transferred to a 5-mL glass vial flushed with N_2 gas and stored at -85˚C until analysis. Approximately 1,000-2,000 eggs were used for fatty acid analysis. Frozen egg samples were thawed and then transferred to three pre-weighed vials and weighed again to determine wet weight. A solution of 1:1 chloroform/methanol was added to each vial and the eggs were transferred to a hand-held, glass homogenizer. Eggs were mechanically homogenized for 5 minutes, then sonicated for 2 minutes, and homogenized for 2 additional minutes. Using a glass Pasteur pipette, the homogenate was transferred to a 25-mL round bottom flask in 1:1 chloroform/methanol. This flask was placed on a rotary vaporizer until all solvent had evaporated and the homogenate appeared dry. The round bottom flask containing the egg homogenate was dried using a vacuum-pump for at least 5 minutes. A mini-filter was made using a 1-cm X 1-cm Kim-wipe© tissue pushed into the bottom of a 10-cm Pasteur pipette. The dried homogenate was transferred to this mini-filter using 1 mL of 1:1

chloroform/methanol and rinsing 3 times into a pre-weighed 25-mL round bottom flask to remove egg shell and other non-lipid parts of the egg. Once again, the egg homogenate was placed on a rotary-evaporator until solvent was removed then dried using a vacuum-pump. The homogenate, free of contaminates, was weighed. Percent lipid was calculated by dividing the weight of extracted lipid by the wet-weight of the egg sample. Using three 1 mL aliquots of 1:1 chloroform/methanol solvent, the homogenate was re-suspended and transferred to a 5-mL conical vial with a stirring magnet. The chloroform was removed under a stream of N_2 gas in a fume hood. Once the solvent had evaporated to approximately 0.5 mL, the vial was removed from the gas and 1 mL of 0.5 M NaOH was added to each conical vial. This solution was placed on a hot-plate stirrer and maintained between 70-100°C for 30 minutes with the stirring magnet continuously mixing the solution. After 30 minutes, 1.5 mL of BF_3-methanol was added and temperature and stirring was maintained for an additional 30 minutes. The solution was removed from the hot-plate stirrer, allowed to cool for approximately 5 minutes, and then 1 mL of saturated salt solution and 1 mL of hexane was added. The conical vial was capped and shaken and the solvent layer was allowed to separate. The hexane layer on top was removed using a 20-cm Pasteur pipette and filtered though a 32-μm silica column. To remove excess water from the lipid solution, a filter column was made by placing a 1-cm X 1-cm piece of Kim-wipe© tissue pushed into the bottom of a 10-cm Pasteur pipette, then filled 3/5 to the top with silica powder. Approximately 1 mL of hexane was added twice to the conical vial and filtered into a clean 25-mL round bottom flask. As a final rinse of the conical vial, 20% ether-hexane was added, mixed, and decanted through the silica filter into the round bottom flask. This solvent solution was

rotary evaporated and dried under a vacuum. Finally, 200 μL of chloroform was added to the round bottom flask with a micro-pipette and dissolved lipid was transferred with a 20-cm Pasteur pipette into a gas chromatography vial, this rinse was performed twice. The gas chromatography vial was flushed with N_2 gas and stored in a refrigerator at 4°C until the sample could be analyzed. Fatty acid methyl esters (FAME) were the end product of the reaction with BF_3-methanol. Samples were run on a Hewlett-Packard gas chromatograph with a starting temperature of 195°C for 5 minutes, followed by a ramp-up for 14 minutes to a final temperature of 270°C at 15°C / min and held for 5 minutes. Chromatograph peaks were identified using FAME standard retention times (GLC-84 Nu Chek Prep U.S.). Relative abundance for each peak was converted to percent abundance for each peak in each sample.

Statistics

Statistics were performed using JMP Start Statistical software, version 4.0.4. (SAS Institite, Cary, NC). For both phosphorus and fatty acid spectra, peak area was converted to percent relative abundance and then arcsine transformed before ANOVA was used to compare differences between eggs of high and low quality or at different stages of embryonic development. Differences between stages of development were determined using Tukey's test for significance.

RESULTS

Phosphorus Composition and Egg Viability

Phosphorus composition was compared in viable and non-viable eggs. In a typical ^{31}P-NMR spectrum from fertilized floating (i.e. viable) southern flounder eggs (blastula stage) a number of peaks were apparent (Fig. 1). The most abundant phosphorus compound was phosphatidylcholine (PC), represented by the largest peak shown. Other peaks included inorganic phosphate (Pi), unidentified phosphate monoesters (Pm), phosphatidylethanolamine (PE), phosphatidylinositol and/or phosphatidylserine (PI/S), creatine phosphate (PCr), nicotinamide adenine dinucleotide phosphate (NADP) and the three phosphates from ATP (Pγ, Pα, and Pβ).

A typical ^{31}P-NMR spectrum for unfertilized floating eggs is shown in Fig. 2. Peaks appeared sharp for all phosphorus compounds seen in fertilized floating eggs (Fig.1). A spectrum for unfertilized sinking (i.e. non-viable) eggs is shown in Fig.3. This spectrum showed an erosion of most peaks when compared to unfertilized and fertilized floating eggs. Pi levels were significantly ($P < 0.01$) higher in sinking eggs (14.2%) than in floating eggs (5.5%). PE levels were significantly ($P < 0.005$) higher in unfertilized floating eggs (6.6%) than in unfertilized sinking eggs (0.0%). PCr and NADP levels were significantly ($P < 0.001$) lower in unfertilized sinking eggs (0%) than in unfertilized floating eggs (3.6% and 1.8%, respectively). The alpha and gamma phosphates of ATP were significantly ($P < 0.01$) higher in floating eggs than in sinking eggs (6.1% and 5.9%, vs. 1.5% and 2.1%, respectively) (Fig. 4).

Intracellular pH and Egg Viability

Using the chemical shift of the Pi peak, intracellular pH (pHi) was determined. In unfertilized sinking eggs, the pHi was maintained at 7.3. In unfertilized floating eggs, the pHi was 7.0. In fertilized floating eggs the pHi decreased from 7.1 (15 h post-spawn) to 6.2 (24 h post-spawn) and then increased to 6.8 (48 h-post-spawn) (Fig. 5).

Phosphorus Composition and Egg Stage

Mean fertilization success, hatching success, and survival to first-feeding for selected batches of high quality eggs and embryos used to compare phosphorus composition at different stages of development are shown in Table 1. Five stages of development were compared: unfertilized, blastula, gastrula, early embryo, and late embryo.

^{31}P-NMR spectra of southern flounder embryos at the gastrula, early embryo and late embryo stages are shown in Figs. 6-8, respectively. The levels of the α-phosphate, β-phosphate and γ-phosphate of ATP showed a clear decrease during development, from highest levels in unfertilized eggs (Fig. 2) to lowest levels in late embryo stages (Fig. 8). Levels of the α-phosphate, β-phosphate and γ-phosphate of ATP were significantly ($P < 0.001$) lower in late embryos than in all earlier stages of development (Figure 9). Pi, PC, PI/S, and PCr levels did not significantly ($P > 0.05$) change over the five stages studied. (Figs. 1-2. and Figs. 6-8).

Phosphatidylethanolamine (PE) progressively increased from low levels in unfertilized eggs to a maximum during the late embryo stage (Fig. 10). PE levels were

significantly (P < 0.001) higher in early to late embryo stages, compared with unfertilized, blastula, and gastrula stages.

Phosphorus Composition and Egg Quality

Phosphorus composition was compared for low and high quality embryos at four stages of development including, blastula, gastrula, early embryo, and late embryo. Table 2 compares fertilization success, hatching success, and survival to first feeding of low and high quality southern flounder eggs used for these comparisons. Overall, mean fertilization success, hatching success, and survival to first feeding for all four stages was 91.4, 74.2, and 55.7% respectively, for high quality eggs, compared to 56.6, 39.7, and 21.4%, respectively, for low quality eggs. For fertilization success, significant (P < 0.001) differences between high and low quality eggs were observed at the blastula and early embryo stages. For hatching success, no significant (P > 0.01) differences between high and low quality eggs were observed at any stage. Survival to the first-feeding stage was significantly (P < 0.01) higher in high than in low quality eggs at all stages of development.

Percent relative abundance of phosphorus compounds for eggs of high and low quality at four stages of development (blastula, gastrula, early embryo and late embryo) are compared in Figures 11-14. There were no significant (P > 0.05) differences in phosphorus composition between eggs of high and low quality at any of these four stages.

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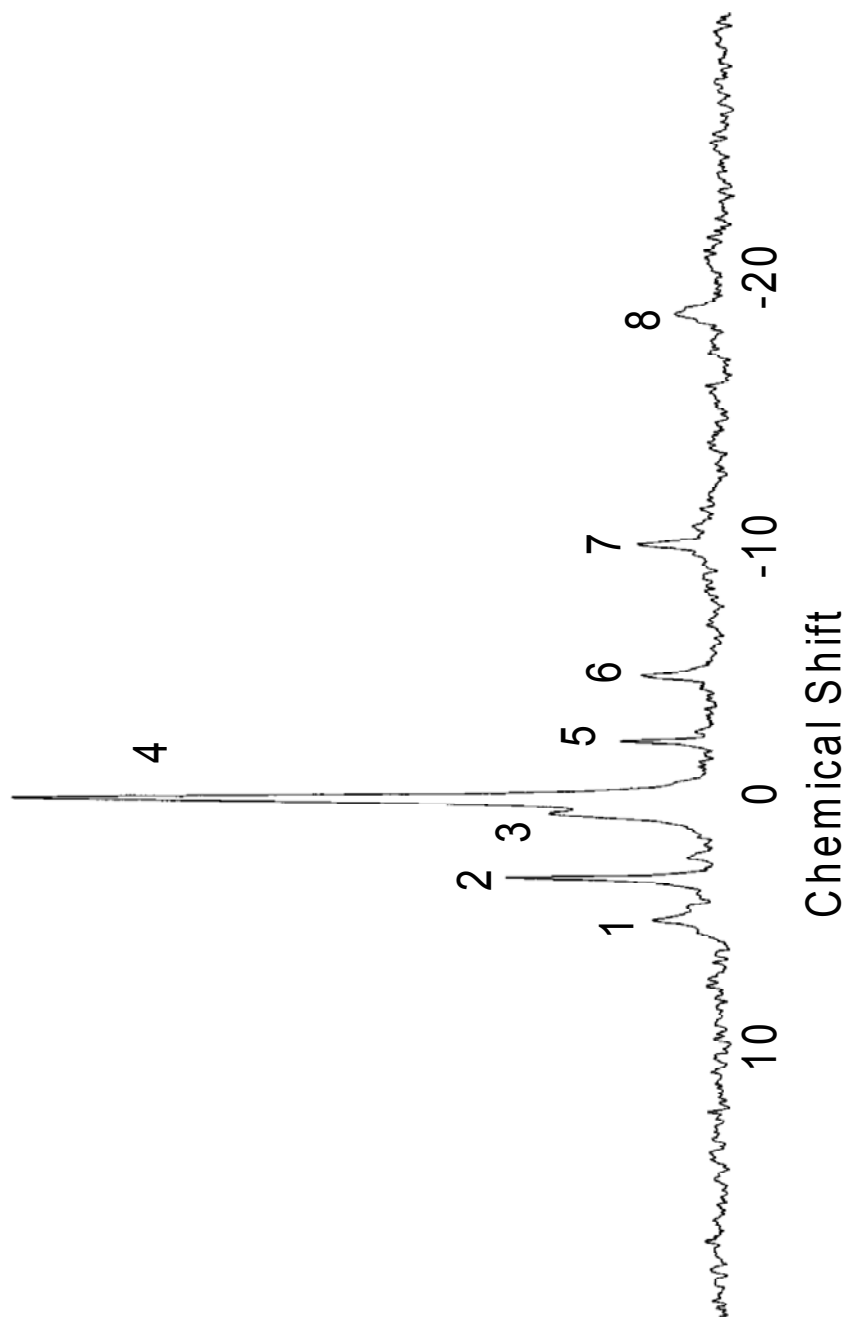
Figure 1. ^{31}P-NMR spectra from fertilized southern flounder embryos at the blastula stage of development (12-16 hours post-fertilization). Major peaks shown from left to right are (1) inorganic phosphate; (2) phosphate monoester; (3) phosphatidylinositol/serine; (4) phosphatidylcholine; (5) creatine phosphate; (6) γ-phosphate ATP; (7) α-phosphate of ATP with underlying phosphate moiety of NAD; (8) β-phosphate of ATP.
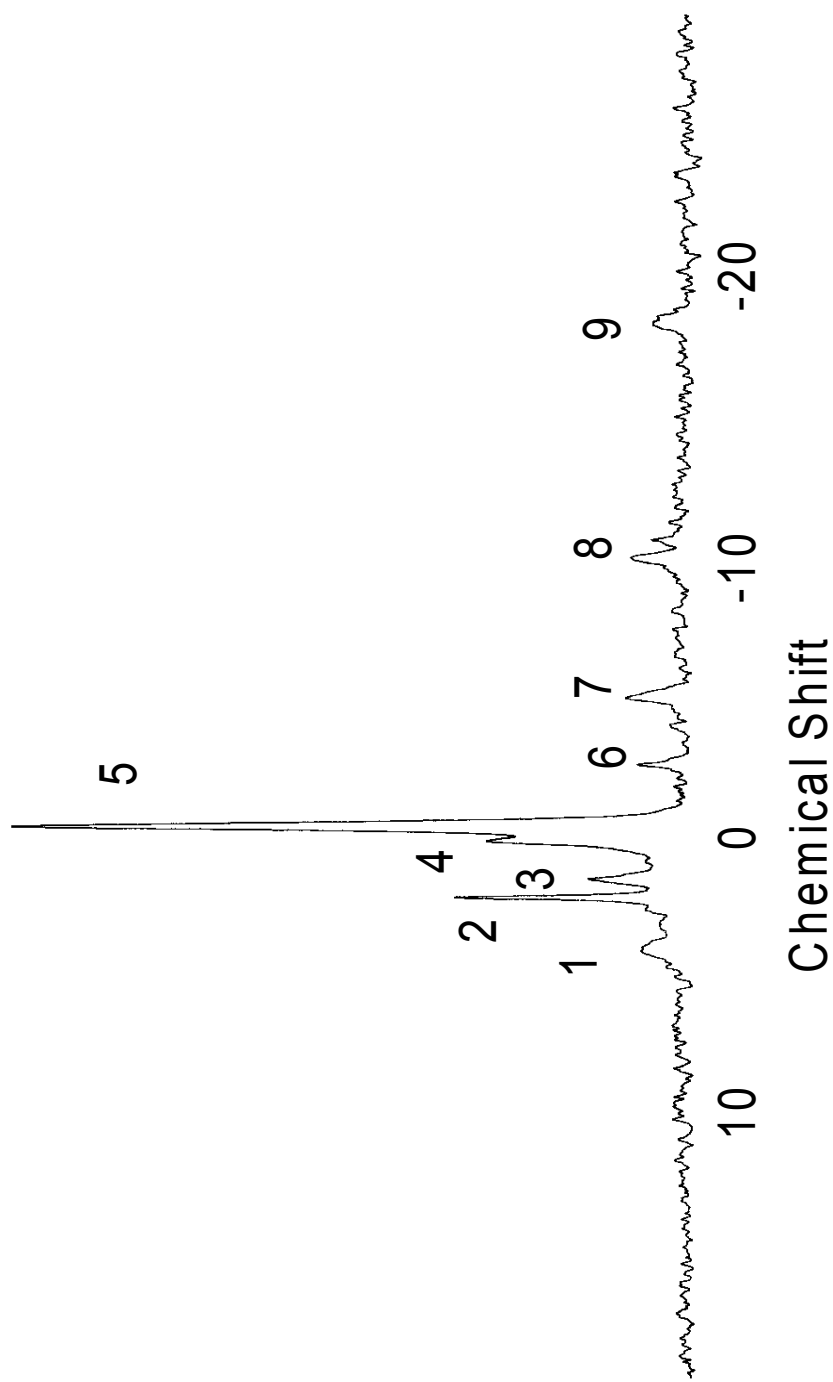
Figure 2. ^{31}P-NMR spectra from unfertilized floating southern flounder eggs. Major peaks shown from left to right are (1) inorganic phosphate; (2) phosphate monoester; (3) phosphatidylethanolamine, (4) phosphatidylinositol/serine; (5) phosphatidylcholine; (6) creatine phosphate; (7) γ-phosphate ATP; (8) α-phosphate of ATP with underlying phosphate moiety of NAD; (9) β-phosphate of ATP.
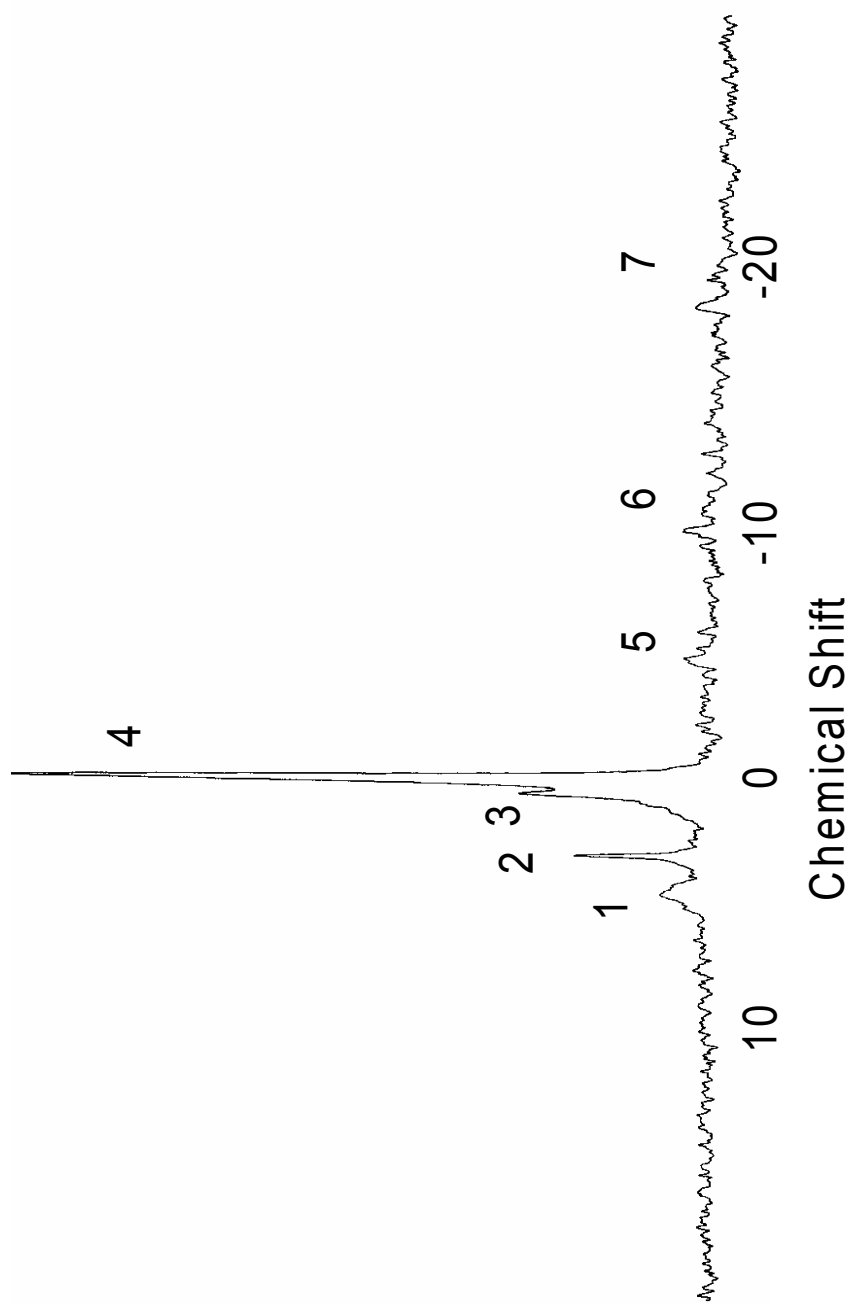
Figure 3. ^{31}P-NMR spectra from unfertilized sinking southern flounder eggs. Major peaks shown from left to right are (1) inorganic phosphate; (2) phosphate monoester; (3) phosphatidylinositol/serine; (4) phosphatidylcholine; (5) γ-phosphate ATP; (6) α-phosphate of ATP with underlying phosphate moiety of NAD, (7) β-phosphate of ATP.
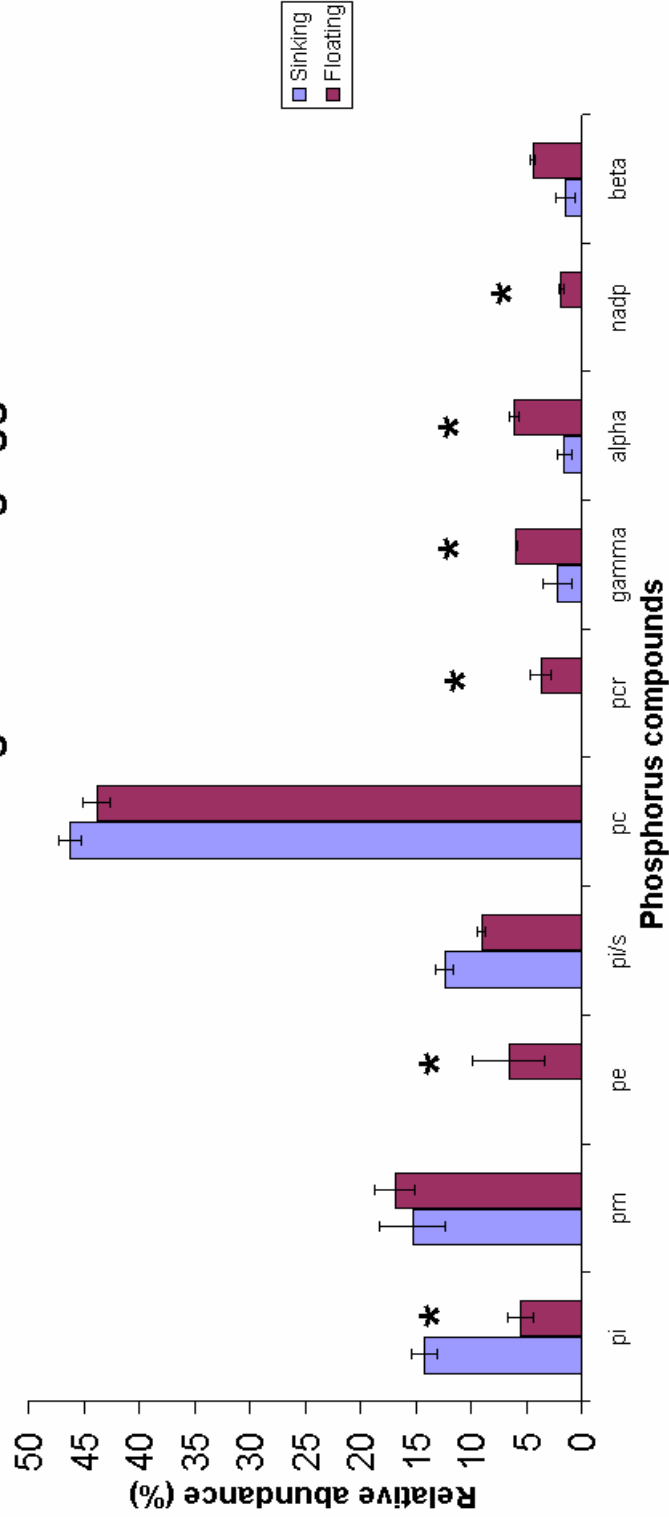
Figure 4. Percent relative abundance of various phosphorus containing compounds for unfertilized floating verses sinking eggs. For each compound, relative abundance was expressed as a percentage of the sum of phosphorus compounds detected in each spectrum. Values represent means ± SE (n = 3). Compounds are abbreviated as follows; inorganic phosphate (pi), phosphate monoester (pm), phosphatidylethanolamine (pe), phosphatidylinositol/serine (pi/s), phosphatidylcholine (pc), creatine phosphate (pcr), nicotinamide adenine dinucleotide phosphate (nadp) and the three phosphates from ATP (alpha, beta, and gamma). Asterisk (*) denotes compounds which are significantly different from each other.
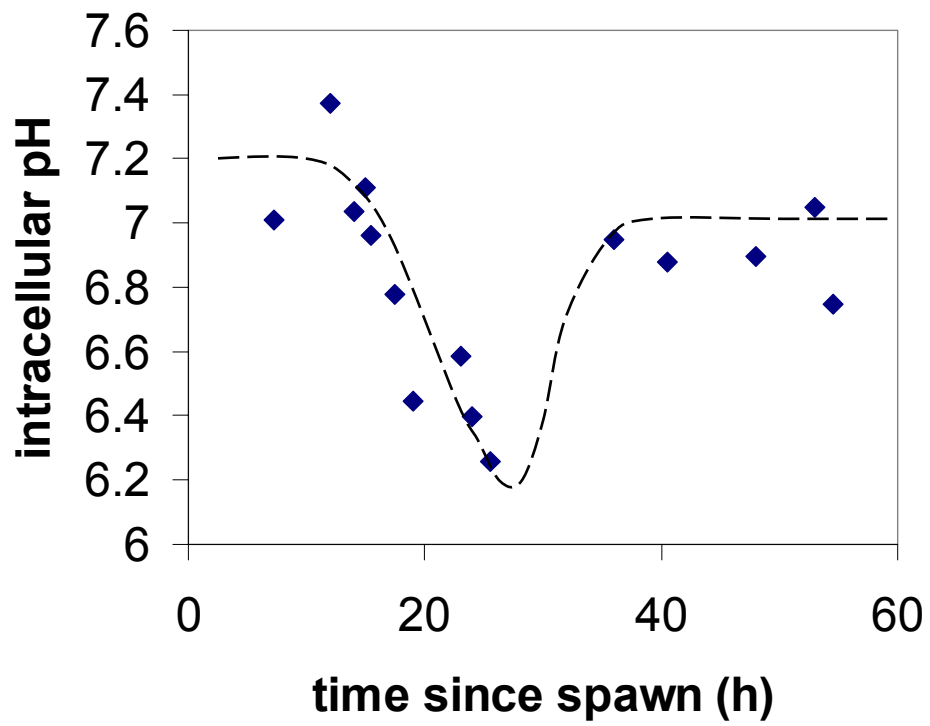
Figure 5. Mean intracellular pH of southern flounder embryos during development.

Table 1. Mean ± SE (n = 3-8) fertilization success, hatching success, and survival to first feeding for selected batches of southern flounder eggs and embryos used to compare phosphorus composition at five stages of development. High quality eggs and embryos were sampled for analysis using a fertilization success (>65%) and survival to first-feeding of (>30%). For unfertilized eggs, multiple batches were sampled prior to artificial fertilization, and then only high quality unfertilized egg samples were used for analysis.

Stage	Fertilization	Hatch	Survival (4-day)
Unfertilized	67.0 ± 11.3	53.2 ±11.9	35.7 ± 12.1
Blastula	93.1 ± 1.9	63.9 ± 4.7	47.2 ± 3.3
Gastrula	91.6 ± 2.9	78.6 ± 6.6	54.6 ± 4.6
Early Embryo	93.5 ± 1.9	77.8 ± 11.5	56.8 ± 5.8
Late Embryo	87.3 ± 10.9	79.6 ± 10.7	63.1 ± 11.7
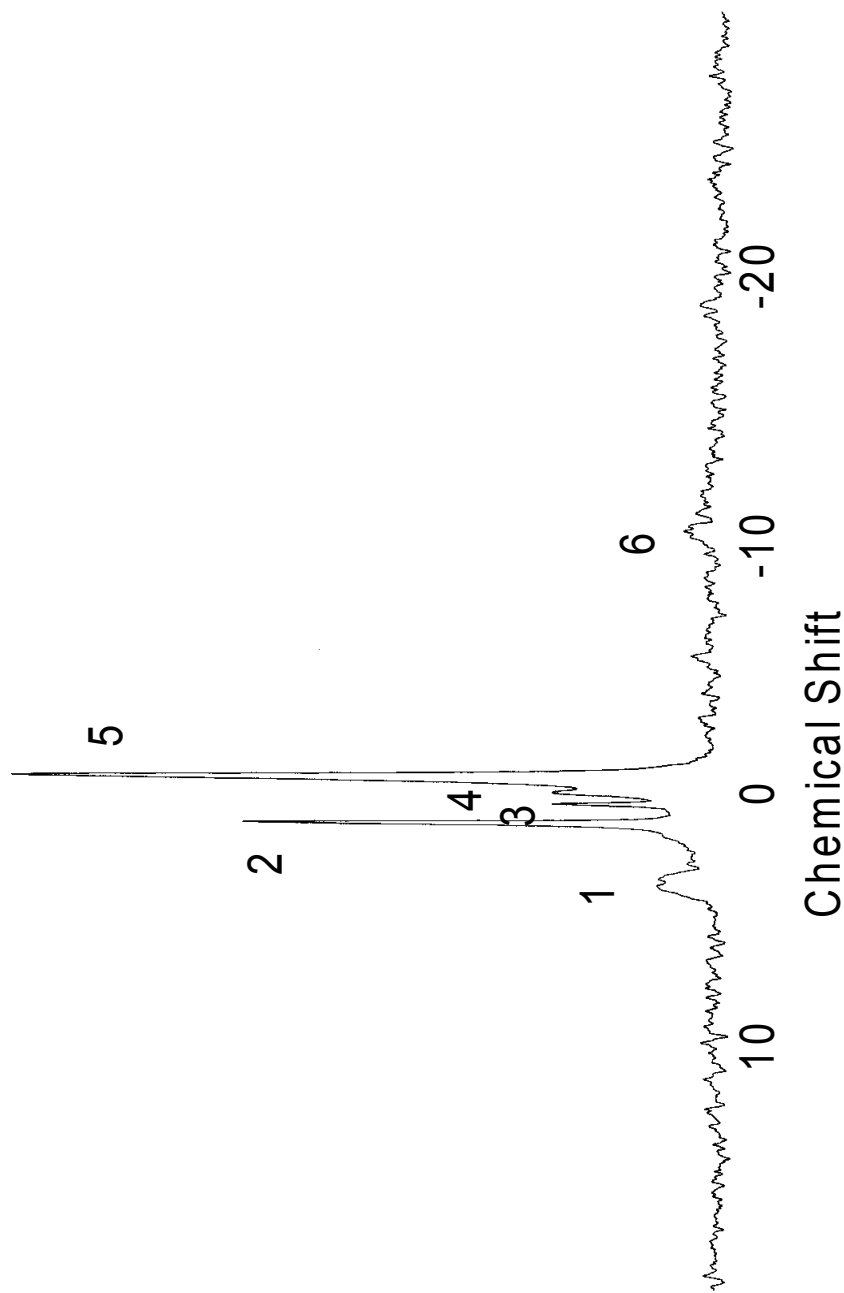
Figure 6. ^{31}P-NMR spectra from fertilized southern flounder embryos at the gastrula stage of development (20-24 hours post-fertilization). Major peaks shown from left to right are (1) inorganic phosphate; (2) phosphate monoester; (3) phosphatidylethanolamine; (4) phosphatidylinositol/serine; (5) phosphatidylcholine; (6) α-phosphate of ATP.
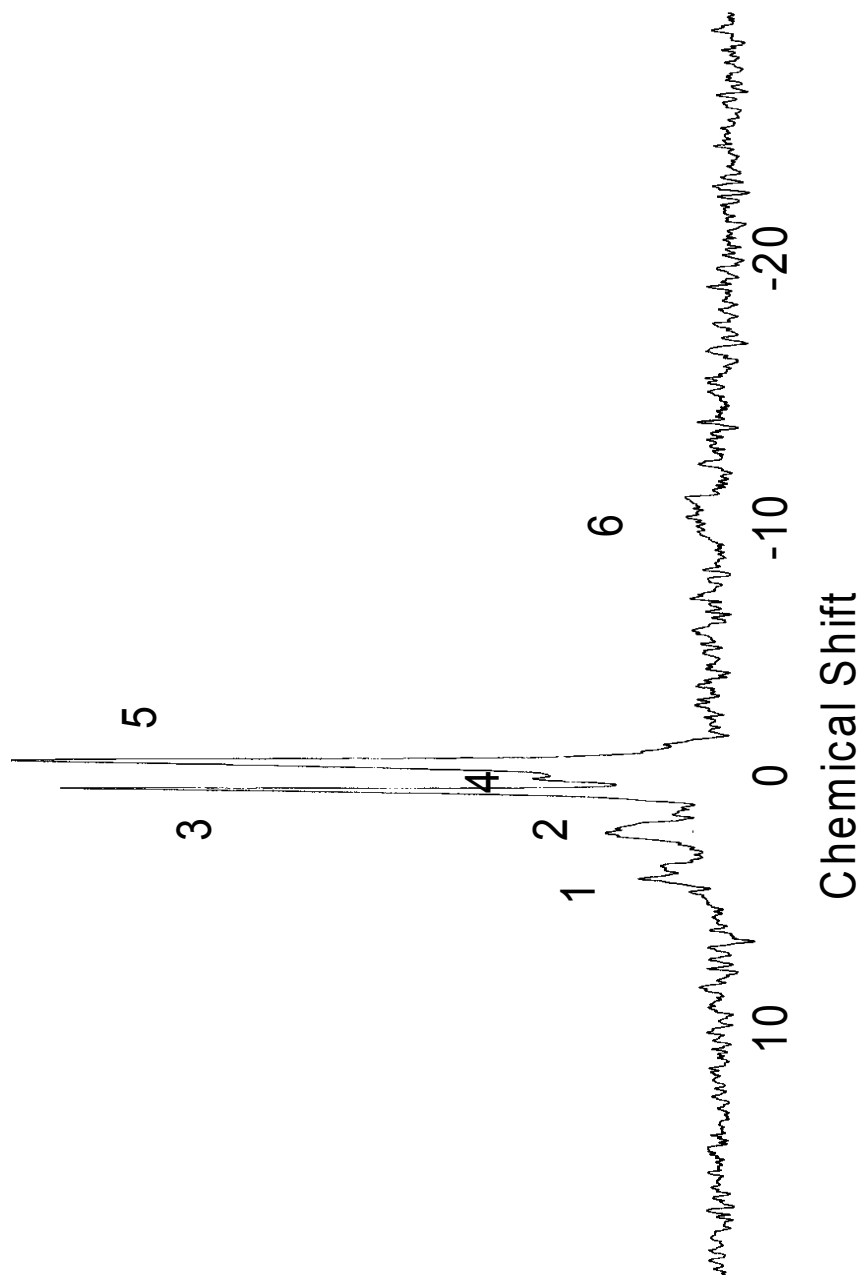
Figure 7. ^{31}P-NMR spectra from fertilized southern flounder embryos at the early embryo stage of development (32-36 hours post-fertilization). Major peaks shown from left to right are (1) inorganic phosphate; (2) phosphate monoester; (3) phosphatidylethanolamine; (4) phosphatidylinositol/serine; (5) phosphatidylcholine; (6) α-phosphate of ATP.
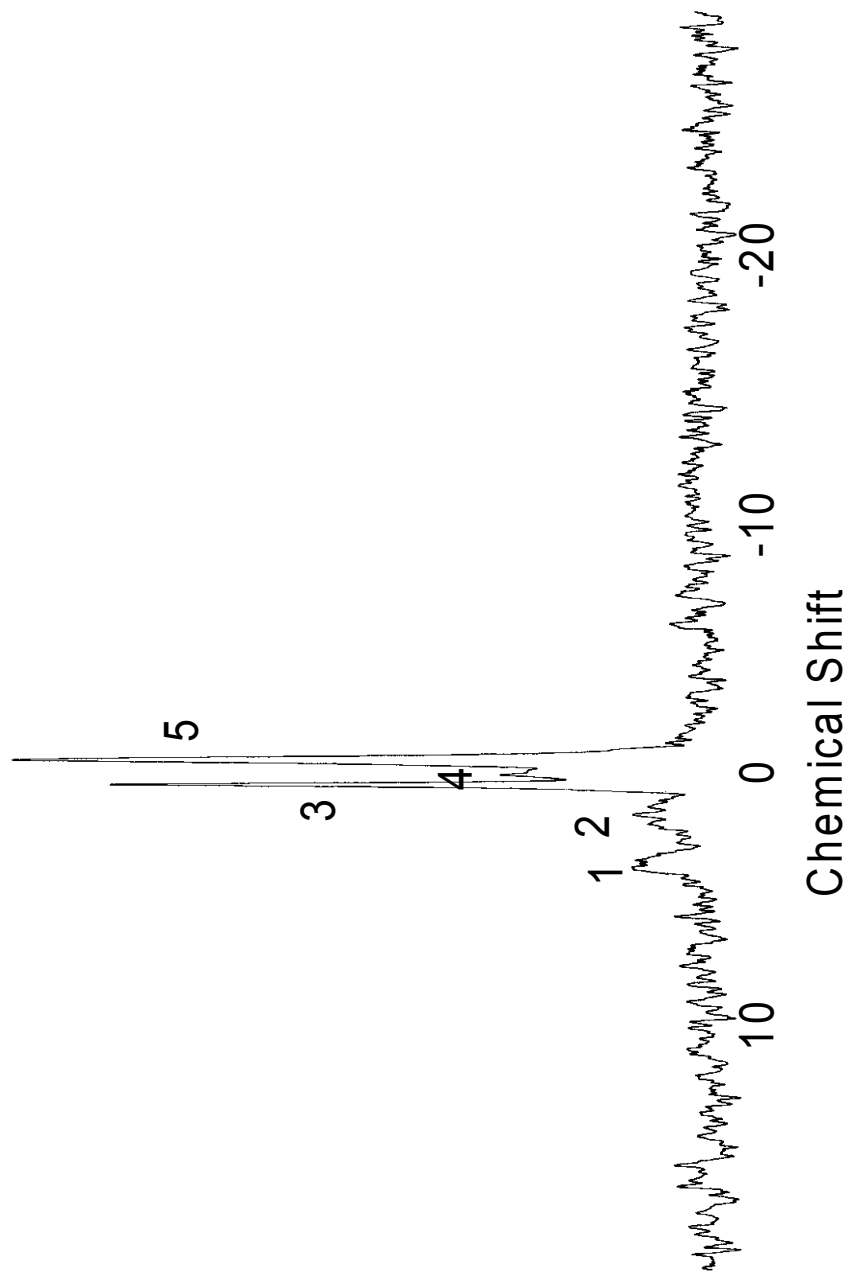
Figure 8. ^{31}P-NMR spectra from fertilized southern flounder embryos at the late embryo stage of development (68-72 hours post-fertilization). Major peaks shown from left to right are (1) inorganic phosphate; (2) phosphate monoester; (3) phosphatidylethanolamine; (4) phosphatidylinositol/serine; (5) phosphatidylcholine
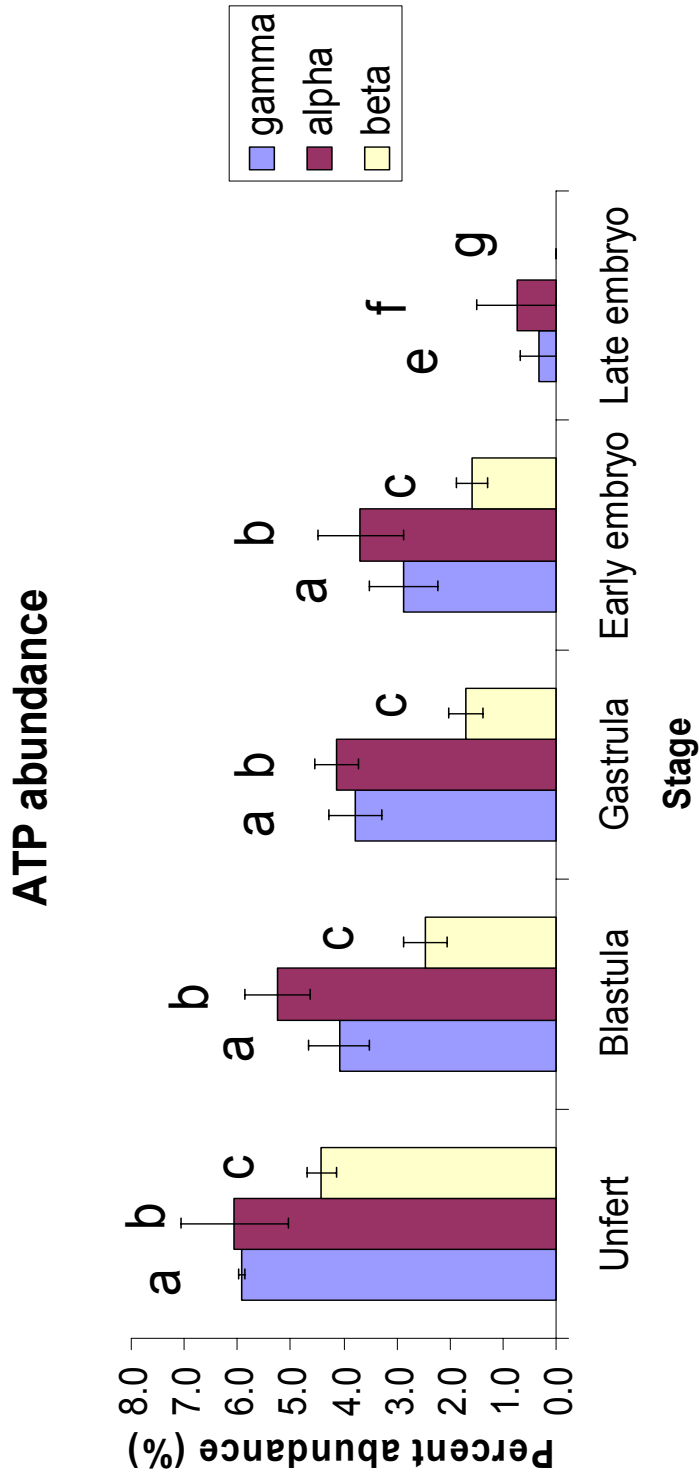
Figure 9. Percent relative abundance of ATP phosphates (gamma, alpha, and beta) in eggs at five stages of development. For each type of ATP phosphate, means not sharing a common letter are significantly ($P < 0.005$) different. Mean values $\pm$ SE.
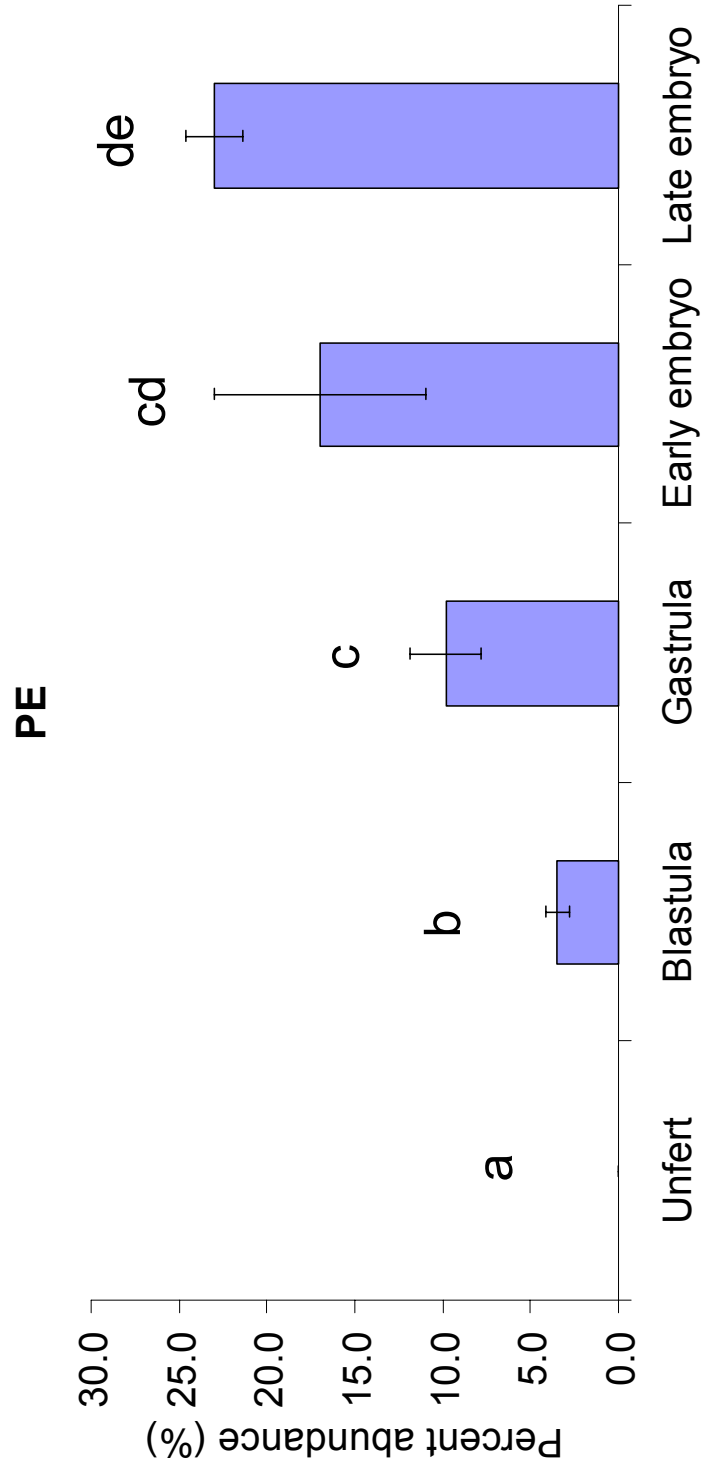
Figure 10. Percent relative abundance of phosphatidylethanolamine (PE) in eggs at five stages of development. Means not sharing a common letter are significantly (P < 0.005) different. Mean values ± SE.

Table 2. Mean ± SE (n = 3-6) fertilization success, hatching success, and survival to first feeding of selected batches of low and high quality southern flounder embryos used to compare phosphorus composition at different stages of development. For each parameter, an asterisk (*) indicates a significant (P < 0.005) difference between low and high quality eggs.

Stage	Quality	Fertilization	Hatch	Survival (4-day)
Blastula	Low	52.5 ± 2.9*	37.8 ± 9.7	22.3 ± 1.1*
	High	93.1 ± 2.5	63.9 ± 10.3	47.2 ± 1.5
Gastrula	Low	56.7 ± 22.5	28.7 ± 1.3*	18.3 ± 1.3*
	High	92.4 ± 3.3	84.7 ± 10.1	68.8 ± 4.8
Early Embryo	Low	50.1 ± 2.7*	39.0 ± 9.6	21.3 ± 1.7*
	High	93.5 ± 1.9	77.8 ± 11.1	56.8 ± 1.8
Late Embryo	Low	67.1 ± 13.7	53.0 ± 10.8	23.6 ± 2.1*
	High	84.0 ± 14.3	79.6 ± 10.7	66.5 ± 8.3
Overall	Low	56.6 ± 6.2*	39.7 ± 4.8*	21.4 ± 0.9*
	High	91.4 ± 2.6	74.2 ± 4.4	55.7 ± 3.8
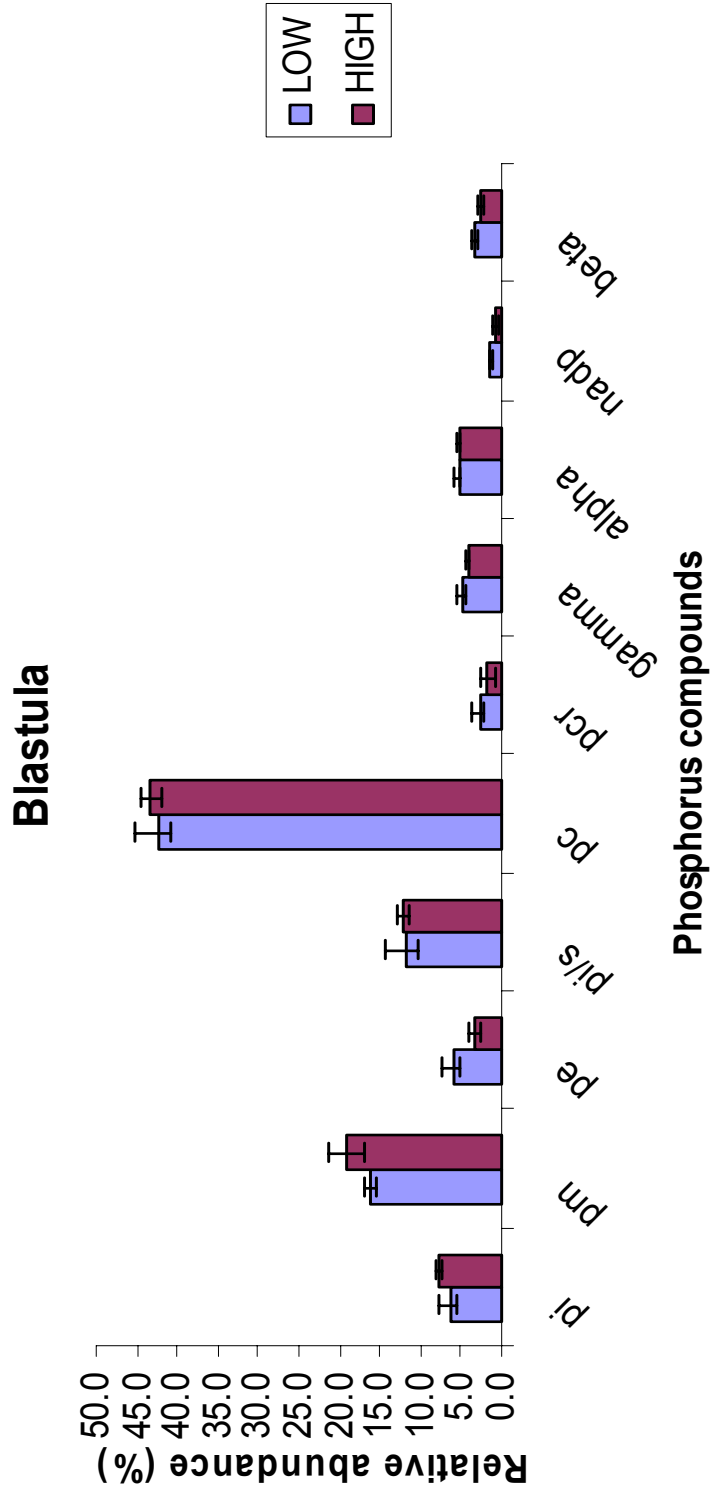
Figure 11. Percent relative abundance of various phosphorus containing compounds for eggs of high and low quality at the blastula stage of development. Values represent means ± SE (n = 6). No significant (P > 0.005) differences between high and low quality eggs were observed for any compound.
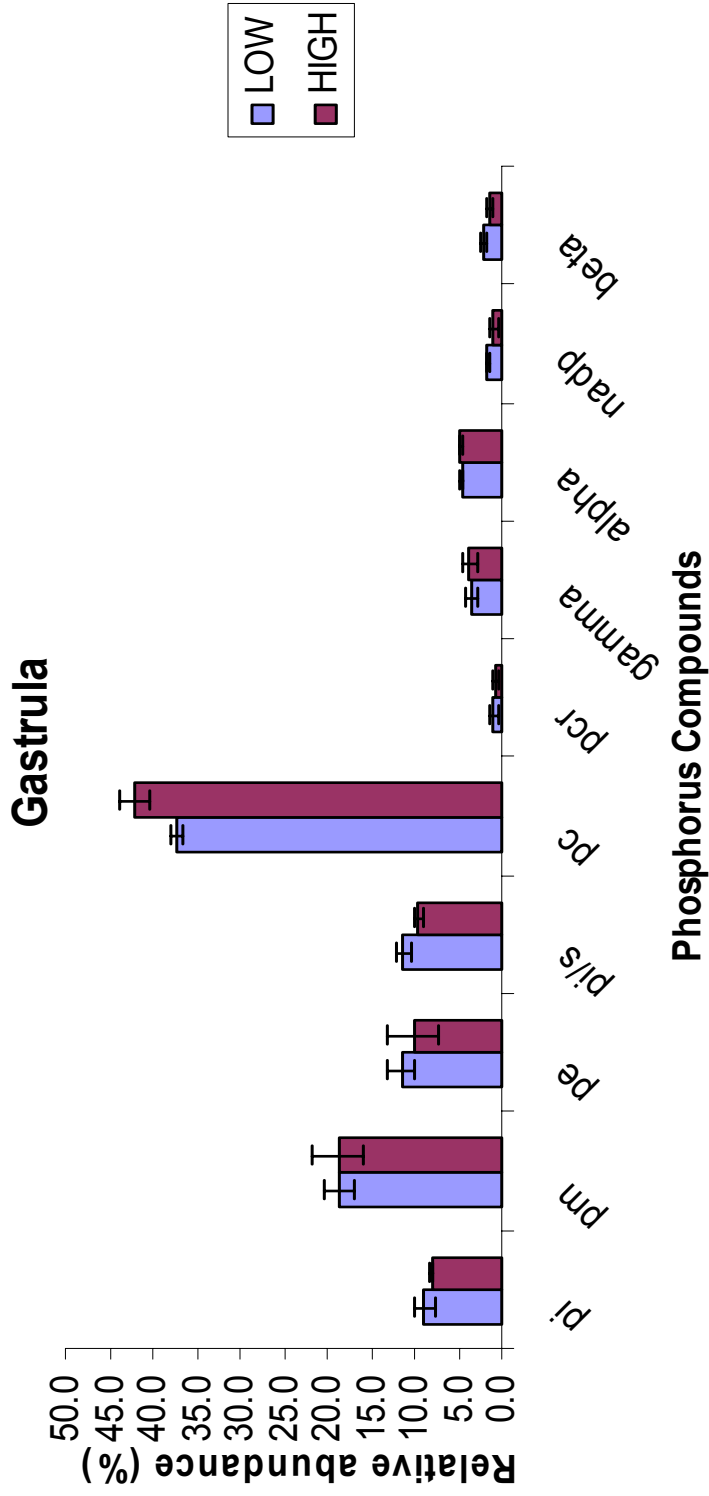
Figure 12. Percent relative abundance of various phosphorus containing compounds for eggs of high and low quality at the gastrula stage of development. Values represent means ± SE (n = 3). No significant (P > 0.005) differences between high and low quality eggs were observed for any compound.
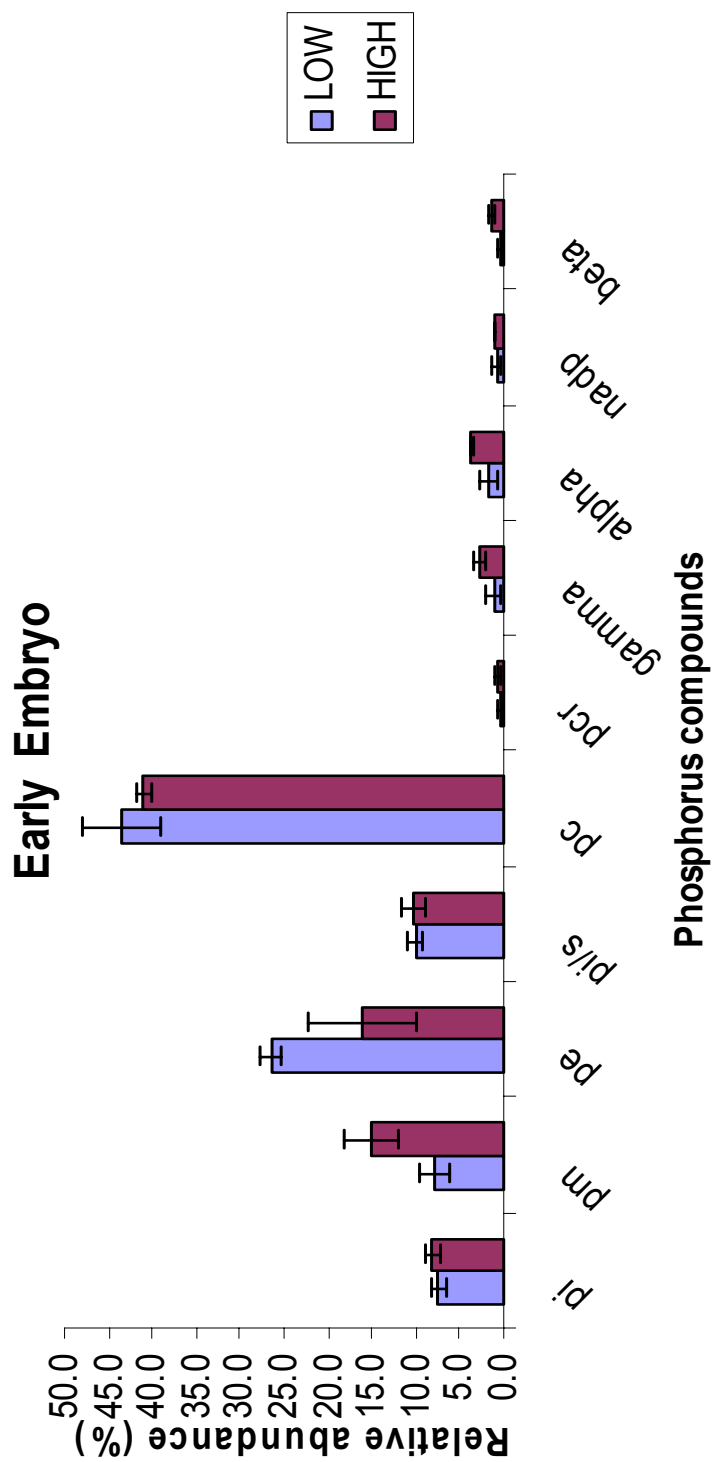
Figure 13. Percent relative abundance of various phosphorus containing compounds for eggs of high and low quality at the early embryo stage of development. Values represent means ± SE (n = 4). No significant (P > 0.005) differences between high and low quality eggs were observed for any compound.
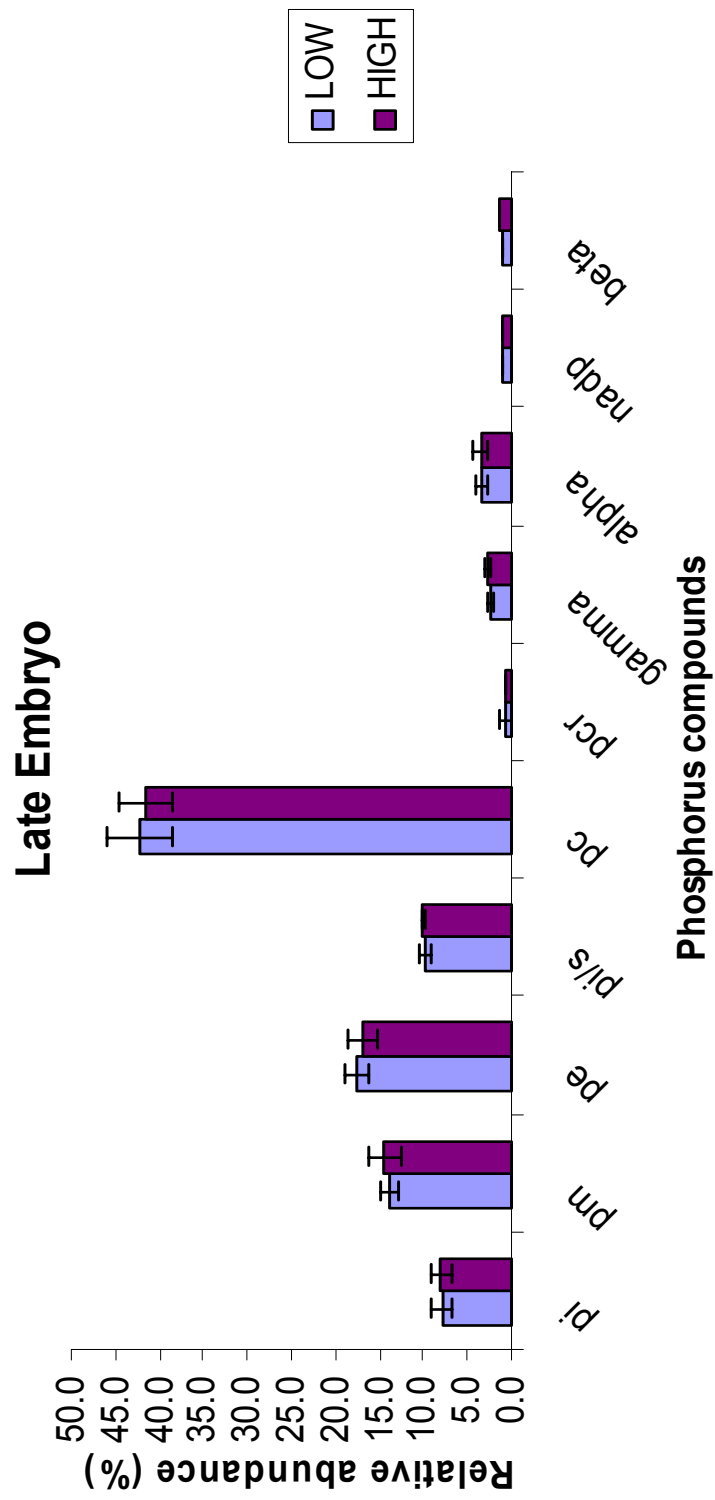
Figure 14. Percent relative abundance of various phosphorus containing compounds for eggs of high and low quality at the late embryo stage of development. Values represent means ± SE (n = 3). No significant (P > 0.005) differences between high and low quality eggs were observed for any compound.

Fatty acid profile of fertilized and unfertilized flounder eggs

A typical GC spectrum of unfertilized flounder eggs is shown in Figure 15. Fatty acids eluted from the column by the length of their carbon chain starting with 14 carbons and ending with 22 carbons. The most abundant fatty acids and their mean percent of total fatty acids included, 16:0 (palmitic acid) at 21.5%, 18:1 n-9 (oleic acid) at 17.5%, and 22:6 n-3 (docosahexanoic acid, DHA) at 26.1%. Other important essential fatty acids included 20:4 n-6 (arachidonic acid, ARA) at 1.9%, and 20:5 n-3 (eicosapentaenoic acid, EPA) at 3.4%.

Figure 16 shows a GC spectrum from fertilized eggs (97.8% fertilization rate) at the blastula stage (12 hours post-fertilization). There were no significant ($P > 0.05$) differences in fatty acid concentrations between unfertilized and fertilized eggs at any stage (Table 3).

Fatty acid profiles and egg quality

To compare fatty acid composition in high and low quality embryos, only blastula stage (approximately 12 hours post-fertilization) embryos were used to minimize differences related to developmental stage. Fertilization success and survival to first feeding of selected batches of southern flounder embryos used to compare high and low quality eggs are shown in Figure 17. High quality spawns had a significantly higher ($P < 0.001$) mean fertilization rate (91.5% vs. 39.0%) and survival to first feeding (64.9% vs. 13.1%) than low quality spawns.

Percent lipid and percent fatty acid of total lipid for low and high quality eggs are compared in Table 4. There were no significant differences ($P > 0.05$) between low and

high quality embryos in percent lipids or in total lipid fatty acid profiles at the blastula stage (Fig.18). For both high and low quality embryos, the percent lipid was 4.4% of wet weight, DHA averaged 26.7%. Levels of EPA averaged 3.3%. The level of ARA averaged 1.9%. Ratio of DHA to EPA averaged 8.3 (Table 5). Table 4 compares the sum of fatty acid classes including saturated, monounsaturated, and polyunsaturated, the sum of and n-6 and n-3 fatty acids, and ratios of n-6 and n-3 fatty acids and ratios of essential fatty acids including DHA:EPA, and ARA:EPA. There were no significant ($P > 0.05$) differences found in any sum or ratios between embryos of high and low quality.

Fatty acid profiles and egg stage

To determine differences in fatty acid composition of total lipid extracts during development, five stages of embryonic development were compared: unfertilized, blastula, gastrula, early embryo, and late embryo. Fertilization success and survival of embryos selected for all stages compared averaged 90.5 and 46.5%, respectively. Percent lipid and percent fatty acid of total lipid for different stages are shown in Table 3. There were no significant differences ($P > 0.05$) between percent lipid and fatty acids among different stages. Sum means of each class of fatty acid, such as saturated, monounsaturated and polyunsaturated fatty acids, as well as sums of n-6 and n-3 fatty acids, and important ratios of fatty acids, such as DHA:EPA, for each stage are shown in Table 6. There were no significant ($P > 0.05$) differences in fatty acid class sums and different stages of embryonic development. A gas chromatograph from the late embryo stage of development (65-72 hours post-fertilization) is shown in Fig. 19 to visually demonstrate no differences between unfertilized (Fig. 15) and blastula stage (Fig. 16).

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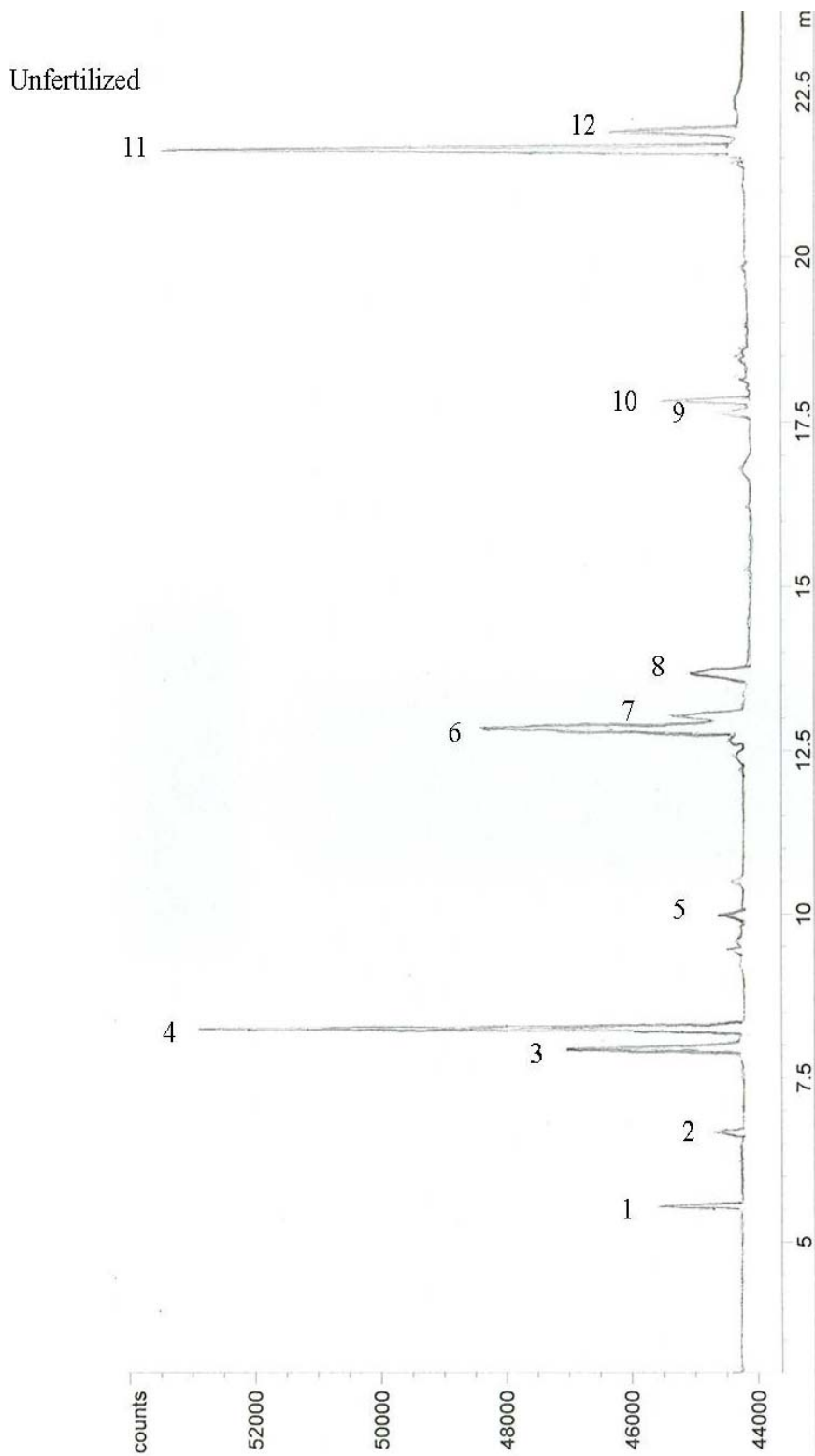
Figure 15. Typical gas chromatograph of fatty acid methyl esters formed from total lipid in unfertilized eggs of southern flounder. Peaks are labeled by their carbon chain length and number of double bonds. Starting from left to right: (1) 14:0 myristic acid, (2) 15:0, (3) 16:1 palmitoleic acid, (4) 16:0 palmitic acid, (5) 17:0, (6) 18:1 n-9 oleic acid, (7) 18:1 n-7, vaccenic acid, (8) 18:0 stearic acid, (9) 20:4 n-6 arachidonic acid, ARA, (10) 20:5 n-3 eicosapentaenoic acid, EPA, (11) 22:6 n-3 docosahexanoic acid, DHA, (12) 22:5 n-3 docosapentaenoic acid.
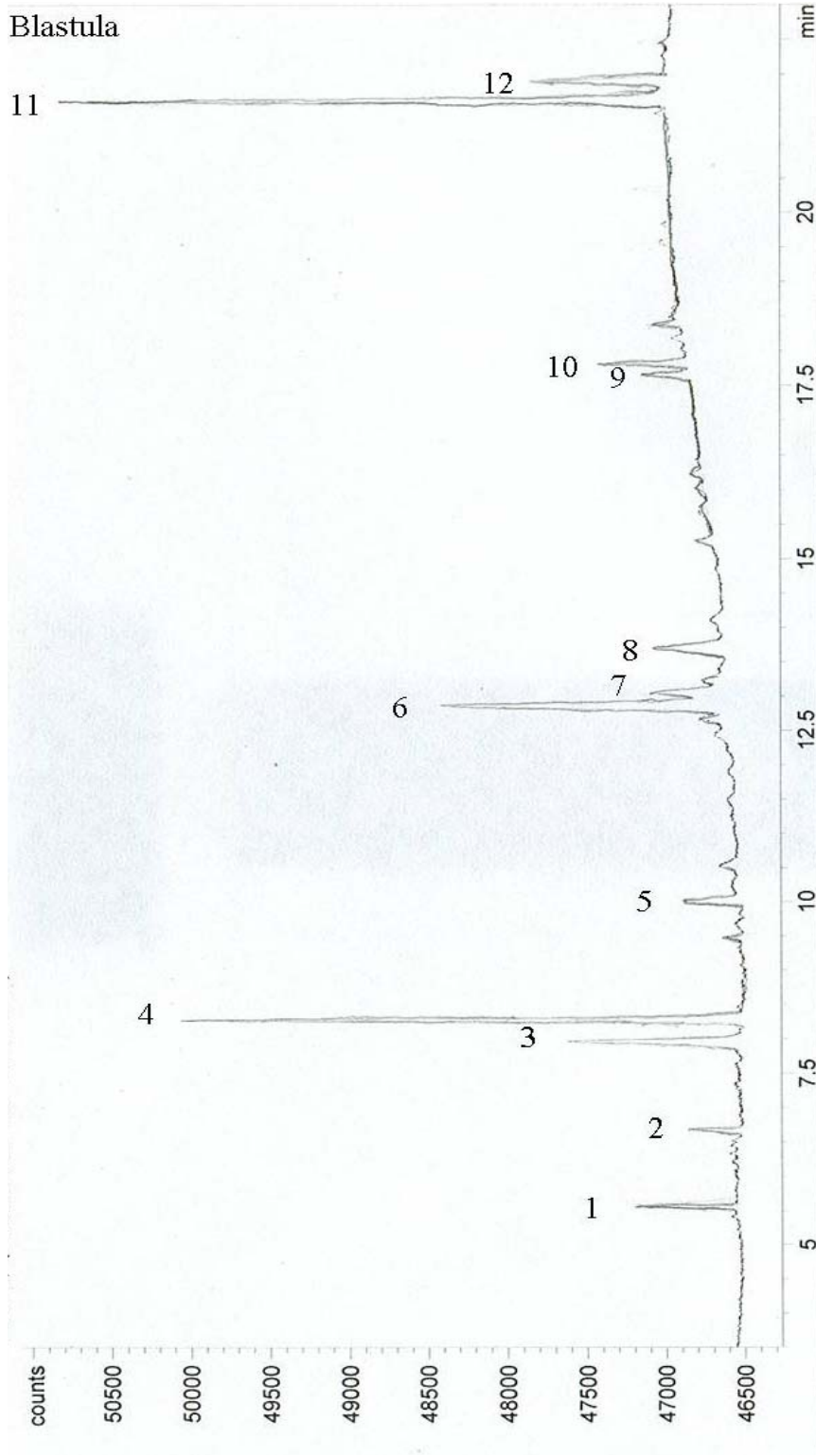
Figure 16. Typical gas chromatograph of fatty acid methyl esters formed from total lipid in fertilized eggs (blastula) of southern flounder. Fertilization rate was 97.8% Peaks are labeled by their carbon chain length and number of double bonds. Starting from left to right: (1) 14:0 myristic acid, (2) 15:0, (3) 16:1 palmitoleic acid, (4) 16:0 palmitic acid, (5) 17:0, (6) 18:1 n-9 oleic acid, (7) 18:1 n-7, vaccenic acid, (8) 18:0 stearic acid, (9) 20:4 n-6 arachidonic acid, ARA, (10) 20:5 n-3 eicosapentaenoic acid, EPA, (11) 22:6 n-3 docosahexanoic acid, DHA, (12) 22:5 n-3 docosapentaenoic acid.

Table 3. Percent lipid and percent fatty acid composition for southern flounder embryos at selected stages development. Values represent means (N = 3) with standard error beside and range below in parentheses.

Parameter	Unfertilized	Blastula	Gastrula	Early Embryo	Late Embryo
% Lipid	3.6 (0.4)	4.6 (0.2)	3.9 (0.2)	4.0 (0.4)	4.4 (0.2)
	(3.2-3.9)	(4.5-4.8)	(3.7-4.0)	(3.6-4.4)	(4.2-4.5)
14:0	2.6 (0.4)	3.1 (0.6)	2.3 (0.3)	2.3 (0.3)	2.4 (0.3)
	(2.1-3.0)	(2.5-3.7)	(2.0-2.6)	(2.0-2.7)	(2.2-2.7)
15:0	1.8 (0.9)	1.7 (0.2)	1.6 (0.3)	1.3 (0.5)	1.7 (0.0)
	(1.0-2.6)	(1.6-2.0)	(1.4-1.9)	(0.9-1.8)	(1.7-1.7)
16:1 n-7	8.5 (0.7)	8.1 (0.4)	7.7 (0.2)	8.0 (0.6)	7.8 (0.4)
	(8.0-7.6)	(7.9-8.6)	(7.5-7.9)	(7.5-8.7)	(7.3-8.0)
16:0	21.8 (2.4)	22.33 (3.2)	18.6 (1.7)	22.9 (3.3)	22.0 (2.5)
	(20.0-25.3)	(19.4-25.8)	(17.3-20.5)	(19.5-26.2)	20.0-24.7)
17:0	2.8 (1.8)	2.7 (0.5)	3.3 (0.5)	2.7 (1.1)	2.9 (0.4)
	(1.3-4.4)	(2.4-3.3)	(2.8-3.6)	(1.4-3.5)	(2.6-3.3)
18:1 n-9	16.3 (1.1)	18.1 (2.1)	17.2 (0.7)	17.6 (2.1)	18.5 (1.1)
	(14.8-17.2)	(15.7-19.4)	(16.6-17.9)	(15.2-19.4)	(17.6-19.7)
18:1 n-7	4.5 (0.4)	4.9 (0.7)	4.3 (0.1)	4.9 (0.8)	5.1 (0.3)
	(3.9-4.9)	(4.1-5.3)	(4.1-4.3)	(4.0-5.6)	(4.8-5.2)
18:0	3.7 (0.1)	4.1 (0.1)	3.4 (0.5)	4.3 (0.9)	4.5 (0.6)
	(3.6-3.8)	(4.0-4.3)	(2.8-3.8)	(3.3-4.9)	(3.8-5.0)
20:4 n-6	2.0 (0.5)	1.8 (0.1)	1.9 (0.5)	2.2 (1.0)	1.6 (0.2)
	(1.6-2.6)	(1.7-1.8)	(1.3-2.4)	(1.6-3.3)	(1.4-1.8)
20:5 n-3	3.9 (0.8)	3.2 (0.5)	3.2 (0.1)	3.7 (0.7)	2.9 (0.3)
	(3.2-5.0)	(2.6-3.6)	(3.2-3.3)	(3.0-4.3)	(2.7-3.3)
22:6 n-3	25.6 (2.5)	23.4 (4.6)	29.2 (4.0)	27.3 (4.6)	25.0 (3.7)
	(23.4-28.5)	(19.3-28.3)	(24.8-32.5)	(22.1-30.8)	(21.0-28.3)
22:5 n-3	6.1 (0.4)	5.4 (0.7)	6.7 (1.1)	5.6 (1.0)	5.6 (0.8)
	(5.6-6.5)	(4.7-5.9)	(5.4-7.6)	(4.8-6.8)	(4.9-6.4)

Table 4. Percent lipid and percent fatty acids for southern flounder embryos of low and high quality at the blastula stage of development. Values represent means (N = 4) with SE beside mean, and range below in parentheses. No significant (P > 0.05) differences were found between treatments.

	Low Quality	High Quality
% Lipid	4.4 (0.4)	4.4 (0.8)
	(4.0-4.8)	(3.7-5.5)
14:0	2.5 (0.4)	2.1 (0.4)
	(1.9-3.1)	(1.6-2.6)
15:0	1.6 (0.9)	0.9 (0.2)
	(1.3-1.9)	(0.0-1.6)
16:1 n-7	8.4 (0.4)	7.9 (0.7)
	(7.6-9.5)	(7.5-8.5)
16:0	20.3 (3.7)	20.8 (1.9)
	(17.9-22.5)	(17.3-25.8)
17:0	2.9 (0.5)	2.9 (0.5)
	(2.3-3.6)	(2.4-3.6)
18:1 n-9	17.4 (1.5)	17.5 (1.4)
	(15.5-19.2)	(15.7-19.4)
18:1 n-7	4.7 (0.5)	4.5 (0.4)
	(4.3-5.2)	(4.1-5.3)
18:0	3.5 (0.6)	3.7 (0.7)
	(2.7-4.2)	(2.8-4.3)
20:4 n-6	2.0 (0.2)	1.7 (0.3)
	(1.6-2.4)	(1.3-1.9)
20:5 n-3	3.4 (0.3)	3.1 (0.4)
	(2.8-3.9)	(2.6-3.3)
22:6 n-3	26.5 (5.3)	26.8 (2.9)
	(22.7-30.0)	(19.3-32.5)
22:5 n-3	6.2 (1.3)	6.1 (0.8)
	(5.4-7.0)	(4.7-7.6)

Figure 17. Percent fertilization success and survival to the first feeding stage for southern flounder eggs of low and high quality (N = 4). For each parameter, means with different letters are highly significant (P < 0.05).

Table 5. Sum of fatty acids by class and fatty acid ratios for southern flounder embryos of low and high quality at the blastula stage of development. Values represent means (N = 5) with SE beside and range below in parentheses. Values represent means (N = 4). No significant (P > 0.05) differences were found between treatments.

	Low Quality	High Quality
SATURATES	31.3 (2.9)	31.0 (3.7)
	(26.8-33.5)	(27.0-35.4)
MONOENES	30.6 (1.7)	29.9 (2.2)
	(28.3-33.0)	(27.6-32.6)
PUFA	38.0 (3.6)	37.6 (6.7)
	(34.0-42.0)	(28.3-44.6)
n-6	2.0 (0.3)	1.7 (0.2)
	(1.6-2.4)	(1.3-1.9)
n-3	36.0 (3.7)	35.9 (6.8)
	(32.2-40.4)	(26.5-43.3)
n-6 / n-3	0.05	0.05
	(0.05-0.06)	(0.03-0.07)
DHA / EPA	7.83	8.73
	(6.3-9.3)	(7.5-10.2)
ARA / EPA	0.58	0.55
	(0.45-0.76)	(0.42-0.68)
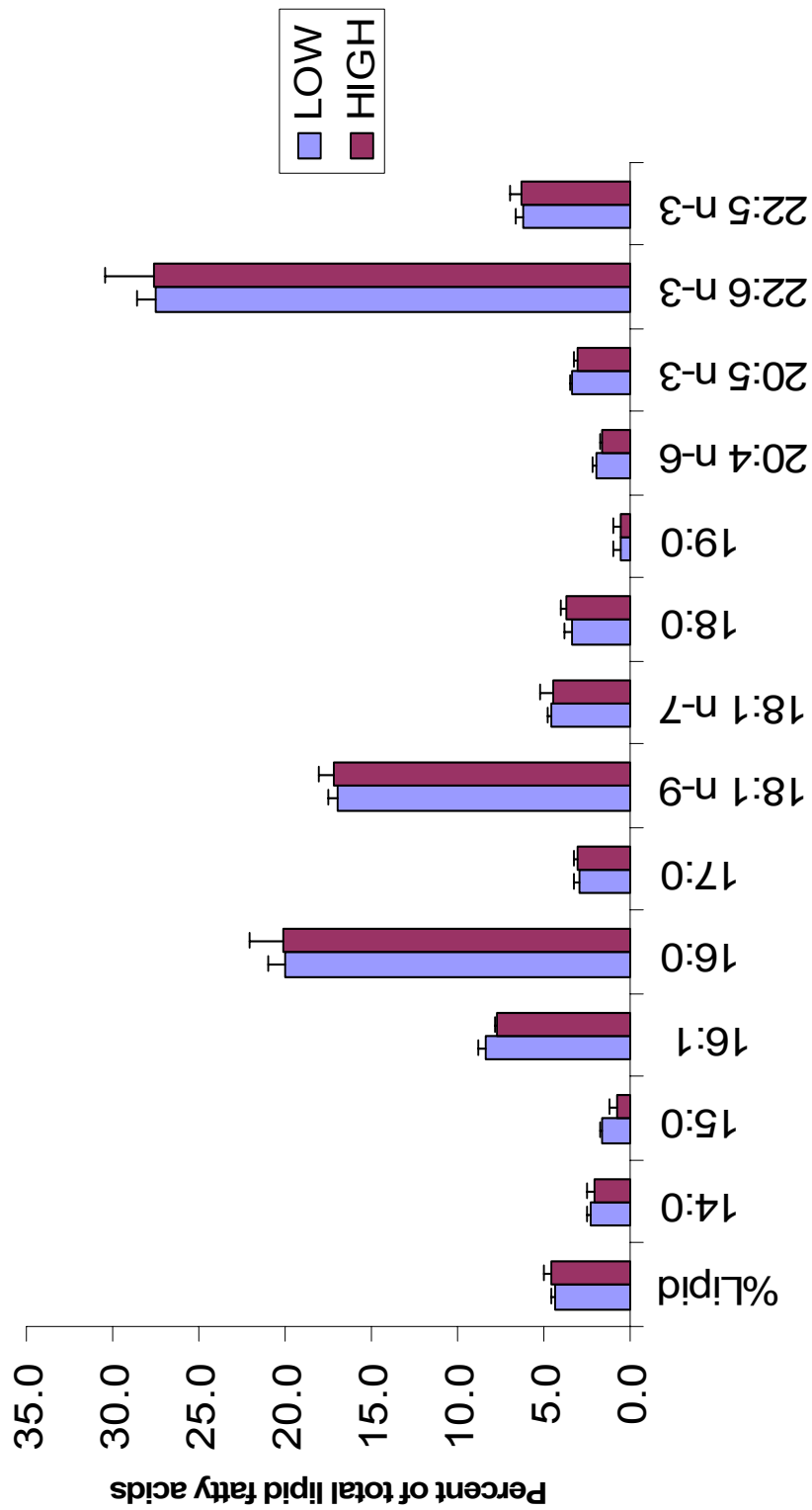
Figure 18. Percent lipid and percent fatty acid of total lipid of low (light bars) and high quality (dark bars) spawns at the blastula stage of development. Values represent means ± SE (N = 5).

Table 6. Sum of total lipid fatty acid classes and important fatty acid ratios for southern flounder embryos at selected stages development. Values represent means with standard error beside and range below in parentheses. N = 4 for unfertilized eggs, and N = 3 for all other stages.

Parameter	Unfertilized	Blastula	Gastrula	Early Embryo	Late Embryo
SATURATES	32.7 (2.1)	34.0 (1.3)	29.3 (0.6)	33.5 (1.2)	33.4 (1.0)
	(29.5-34.2)	(30.0-39.1)	(27.0-32.4)	(29.5-36.2)	(31.5-36.7)
MONOENES	29.4(0.5)	31.1 (0.9)	29.2 (0.3)	30.4 (0.8)	31.3 (0.4)
	(28.7-29.9)	(27.6-33.0)	(28.4-30.2)	(27.1-33.6)	(29.7-32.9)
PUFA	37.6 (2.4)	33.8 (2.1)	41.0 (1.8)	38.7 (1.9)	35.2 (1.7)
	(37.4-40.6)	(28.3-39.0)	(35.8-44.6)	(34.6-43.5)	(30.3-38.9)
n-6	2.0 (0.5)	1.8 (0.1)	1.9 (0.5)	2.2 (1.0)	1.6 (0.2)
	(1.6-2.6)	(1.7-1.8)	(1.3-2.4)	(1.6-3.3)	(1.4-1.8)
n-3	35.6 (2.5)	32.0 (2.3)	39.1 (2.0)	36.6 (2.2)	33.6 (1.8)
	(33.0-39.0)	(26.5-37.3)	(33.4-43.3)	(31.3-41.8)	(28.5-37.2)
n-6 / *n*-3	0.06	0.05	0.05	0.06	0.05
	(0.04-0.08)	(0.05-0.06)	(0.04-0.06)	(0.05-0.08)	(0.05-0.05)
DHA / EPA	6.48	7.41	9.08	7.32	8.56
	(4.7-8.3)	(5.4-10.9)	(7.5-9.8)	(5.1-10.3)	(6.4-10.5)
ARA / EPA	0.50	0.56	0.58	0.58	0.55
	(0.39-0.56)	(0.5-0.7)	(0.4-0.8)	(0.4-1.1)	(0.4-0.7)

Figure 19. Gas chromatograph of fatty acid methyl esters formed from total lipid in (98.4% fertilized) late embryo staged (65-72 hours post-fertilization) embryos of southern flounder. Peaks are labeled by their carbon chain length and number of double bonds. Starting from left to right: (1) 14:0 myristic acid, (2) 15:0, (3) 16:1 palmitoleic acid, (4) 16:0 palmitic acid, (5) 17:0, (6) 18:1 n-9 oleic acid, (7) 18:1 n-7, vaccenic acid, (8) 18:0 stearic acid, (9) 20:4 n-6 arachidonic acid, ARA, (10) 20:5 n-3 eicosapentaenoic acid, EPA, (11) 22:6 n-3 docosahexanoic acid, DHA, (12) 22:5 n-3 docosapentaenoic acid.

DISCUSSION

Phosphorus composition and egg viability

Major contributors to the NMR spectrum of good quality, fertilized southern flounder eggs were inorganic phosphate, PC, and PE in agreement with a similar study on plaice (*Pleuronectes platessa*) eggs (Grasdalen and Jorgensen, 1985). PE was significantly higher in unfertilized floating (i.e. viable) southern flounder eggs than in unfertilized sinking (i.e. non-viable) eggs. In contrast, there were no differences in PE content between viable and nonviable eggs of halibut (*Hippoglossus hippoglossus*) (Bruce et al., 1993). In southern flounder, there were no differences in PC levels between viable and nonviable eggs, in agreement with what was found in viable and nonviable eggs of halibut (Bruce et al., 1993). This could be related to the shared maternally derived lipid source between these two types of eggs.

In this study, PCr, inorganic phosphate, ATP, and NADP were significantly lower in non-viable southern flounder eggs. When viable and nonviable eggs of gilthead sea bream (*Sparus aurata*) were compared, levels of ATP in nonviable eggs were also significantly decreased (Lahnsteiner and Paternello, 2003). In southern flounder, eggs that sank soon after spawning were typically over-ripe, characterized by an opaque color and often misshapen. These sinking eggs were atretic and endogenous compounds associated with normal metabolic activity had begun to degrade prior to their release.

ATP was rapidly depleted in Chinook salmon (*Oncorhynchus tshawytscha)* eggs activated by seawater regardless of fertility, whereas non-activated eggs did not exhibit this decrease (Wendling, 2000). In these eggs, ATP levels decreased significantly 2-h post-fertilization; however, since the extent and timing of ATP decline was the same

(about 25%) in fertilized eggs and unfertilized eggs exposed to seawater, this decrease was believed to be related to seawater activation and independent of fertilization. In Chinook salmon, the chorion water hardening reaction was ATP-dependent and thus, energy-dependent (Wendling, 2000). The authors suggested that ATP was probably used to actively transport free amino acids from inside the vitelline envelope surrounding the embryo to form the protein matrix of the outer chorion. Chorions removed from unfertilized medaka (*Oryzias latipes*) eggs hardened when exposed to a Ca^{2+} solution via an enzyme-driven transglutaminase reaction which formed a cross-linked protein matrix (Iuchi, 1995). Once spawned, the egg is exposed to an osmotic environment different from the ovary. ATP could also be used to power ion-exchange pumps to maintain the osmotic differences between the inside and outside of the chorion during hardening. However, in our study we did not test non-water-activated eggs during this period of chorion hardening to see if there was a net loss of ATP.

In high quality southern flounder embryos, 30-36 hours were required for ATP levels to be significantly reduced after fertilization. At this time the membrane structure of the developing embryo is being better organized. This includes ATP being bound to protein channels in this structure. This does not mean that ATP was not being utilized prior to this reduction. ATP is also produced during metabolism to provide energy for protein formation and for intracellular machinery, such as mitochondria. However, ATP bound within this machinery and protein channels would not be visible in NMR spectra, only ATP which is freely tumbling in the cytoplasm would be visible. Eventually, most free ATP would be tied into energy requiring processes in the developing embryo, causing a net loss of ATP in NMR spectra.

Intracellular pH and egg viability

In southern flounder eggs, pH was 7.1 at the blastula stage (12 hours post-fertilization), decreased to 6.3 at the gastrula stage (24 hours post-fertilization), then recovered and stabilized at 6.8 in early embryos (35 hours post-fertilization). This is similar to what was observed in plaice eggs where the internal pH was initially 6.2 (unfertilized and 1 hour post-fertilization), decreased to 5.3 at the gastrula stage (3 days post-fertilization), then increased to 7.1 in hatchlings (12 days post-fertilization), although no data was reported for the early embryo stage (Grasdalen and Jorgensen, 1985). These authors compared NMR spectra of living eggs to pH values of homogenates (measured by a pH meter) and found these values to be in rough agreement. They suggested that internal pH could be compartmentalized among several sub-cellular environments, with NMR spectra of living eggs and pH values of homogenates representing average cellular values. For example, metabolic nitrogenous waste could accumulate inside of the vitelline space (i.e. in the yolk). This could cause a decrease in pH within the egg until formation of active transporters within the chorion-vitelline membrane surrounding the yolk is sufficient to increase the pH by transporting this waste outside of the chorion.

ATP levels during southern flounder embryonic development showed an inverse relationship with pH. ATP levels did not start to decline until pH reached its minimum value. The subsequent rise in pH could be associated with ATP-mediated transport of metabolites. This relationship supports a transporter theory, where ATP could be is used to stabilize embryonic pH through active transport of nitrogenous waste, such as

ammonia, outside of the developing embryo. It has been shown for Atlantic halibut (*Hippoglossus hippoglossus*), turbot (*Scopthalmus maximus*), and lemon sole (*Microstomus kitt*) that ammonia is accumulated and excreted during embryogenesis (Ronnestad and Fyhn, 1993). This waste formation could help explain the pH drop in early developing southern flounder embryos. Nitrogenous waste compounds are formed from catabolism of free amino acids and protein in southern flounder embryos. To better understand development energetics, free amino acid profiles and measurements in developing embryos are needed.

Phosphorus composition and embryonic stage

In southern flounder, a small PE peak was seen in unfertilized eggs, however, in plaice, the PE peak was not visible until 2 hours after fertilization (Grasdalen and Jorgensen, 1985), indicating species-specific differences in endogenous concentrations of phosphorus compounds.

A significant increase in PE was found in developing southern flounder embryos. It is possible that PE is synthesized or released from an immobile storage form such as yolk vitellogenin, a phospholipoprotein with a complex molecular structure, and is not detected by NMR until its release. Only compounds which are freely tumbling in the cytoplasm and not membrane bound are visible by NMR. Vitellogenin is a maternally derived complex phospholipoprotein that contains approximately 80% protein and 20% lipid (Sargent, 1995a). Sargent (1995a) linked PE formation and PE's relatively high proportion of DHA as a way to conserve DHA being released from PC. It has been shown in halibut larvae that, DHA, released from neutral lipid and PC, was retained by

PE, and that PE has an important role in membrane structure of neural and visual tissues (Ronnestad, 1995). Neural tissue, especially visual structures, can form a large proportion of the total body mass of embryonic and larval fish, so that a large portion of the total DHA required for growth is directed towards cell membrane formation vital for normal visual and neural development (Sargent, 1995a). PE can exist as di-22:6 (n-3), when there are two DHA chains and one ethanolamine linked to a glycerol backbone. Of PE found in the retina of trout and cod, this species accounts for over 40% and over 70%, respectively (Bell and Dick, 1991). In southern flounder embryos, the relatively high build up of PE derived from yolk vitellogenin before hatching could also be related to development of visual structures such as the retina which have a high amount of DHA and are developed after hatching.

In southern flounder embryos, PE levels increased during development, while relative abundance of PC did not change significantly, in agreement with what was observed in embryos of turbot (*Scophthalmus maximus*) (Rainuzzo et al., 1992) and Senegal sole (*Solea senegalensis*) (Vazquez et al., 1994). However, in cod (*Gadus morhua)* PC levels decreased, while PE levels increased during embryonic development (Frasier et al., 1988).

In southern flounder, the ratio of PC to PE decreased during development, starting from approximately 8:1 in unfertilized high quality eggs and gradually decreasing to 2.5:1 in pre-hatching embryos. This is similar to what was found in cod, where PC/PE ratio was 8:1 at fertilization and decreased to 4.8:1 in newly hatched larvae (Frasier et al., 1988). In southern flounder, this decrease in the ratio of PC/PE during embryogenesis was related primarily to an increase in PE. PC, which remained relatively stable and was

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not significantly catabolized during embryogenesis, may be conserved for later larval development. In contrast, in cod embryos, the decrease in PC/PE ratio was due to the catabolism of PC, as well as an increase in PE.

Higher depletion of PC demonstrated in cod embryos than in southern flounder, turbot, and Senegal sole may be species specific and related to a genetically programmed catabolic substrate oxidation sequence. It was shown in cod embryos that levels of DHA (22:6 n-3) increased and that DHA was conserved from PC catabolism and incorporated into PE, which showed a corresponding slight increase as PC decreased (Frasier et al., 1988). In southern flounder embryos, DHA and PC levels did not decrease as PE levels increased. PC could be restructuring itself and releasing DHA to help form PE. To understand what is occurring energetically and structurally during development in southern flounder embryos, all sources of energy, including amino acids and glucose, must also be taken into account. Glucose can be formed from amino acids (for energy) using a pathway called gluconeogenesis. This occurs during the citric acid cycle where free amino acids are converted to glucose, a waste product of which is carbon dioxide. Carbon dioxide is an acidic oxide and reacts with water to give carbonic acid. This probably also plays a role in pH reduction during embryogenesis. Lipids are also formed from amino acids by lipogenesis. Cod are cold water species with long developmental periods and require a high amount of DHA and other long-chain polyunsaturated fatty acids to maintain cell membrane fluidity in cold temperatures, which may explain why over 70% of cod retina PE is di-22:6 (n-3). Cod embryos are able to utilize high maternally derived levels of DHA being released from PC breakdown possibly as an adaptation to cold temperature. PC is also the source of a majority of free inorganic

phosphate used for high-energy nucleotide development and choline used for methyl

metabolism and neurotransmission (Tocher et al., 1985a; Frasier et al., 1988; Sargent

1995a; Rainuzzo et al., 1997). While increasing levels of PE could also be a storage form

for DHA in southern flounder, the source of DHA is not known since there was no

corresponding decrease in PC. Most likely, DHA was at a primary level in the egg at the

beginning of embryogenesis because of maternally derived nutrition.

It has been shown that embryos with oil globules (e.g. southern flounder and

turbot) differ from eggs without oil globules (e.g. cod) in catabolic substrate oxidation

sequence, with glycogen and amino acids serving as the main source of energy for eggs

with oil globules, and amino acids and neutral lipids, in equal proportions, for those

without oil globules (Finn, 1996). In turbot embryos, which contain an oil-globule, the

order of substrate catabolism is as follows: glycogen dependence until 18-19 hours post-

fertilization (blastula), then free amino acids (84%) together with a small amount of PC

(9%) and later wax esters (5%) comprising the fuels of embryogenesis then lipid after

hatching (Finn, 1996). It was shown during embryogenesis in the common dentex,

Dentex dentex, also with a single oil droplet, that the sequence of catabolic substrate

oxidation started with carbohydrate, possibly glycogen, then protein, possibly FAA. It

was not until after hatching that the oil globule was consumed as the dominant fuel

(Mourente et al., 1999). It is possible southern flounder embryos follow a similar pattern,

as evidenced by the lack of significant decreases in PC and fatty acids over the course of

embryogenesis. This further suggests that another component, such as free amino acids

or carbohydrate, must account for metabolic energy needs in southern flounder embryos.

In contrast, in embryonic cod, plaice, and Atlantic halibut, which lack an oil globule, PC

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catabolism during embryogenesis was significant and related to metabolic energy needs and a high requirement for DHA for larval membrane formation. Catabolism of PC also releases inorganic phosphate used for nucleic acid synthesis and choline used in neurotransmission pathways (Rainuzzo et al., 1997).

Fatty acid composition of fertilized and unfertilized eggs

There were no significant differences in fatty acid composition of fertilized or unfertilized southern flounder eggs. The most abundant fatty acids in high quality southern flounder eggs included the saturated fatty acid, palmitic acid 16:0 (21.5%), the monounsaturated fatty acid, oleic acid 18:1 n-9 (17.5%), and the polyunsaturated fatty acid, docosahexanoic acid (DHA) 22:6 n-3 (26.1%). Similar studies of a variety of marine finfish species demonstrate species specific differences in quantities of these fatty acids (Table 7). For example, in freshly stripped spotted wolf-fish *Anarhichas minor* eggs, the most abundant fatty acids were 16:0 (13.5%), oleic acid 18:1 n-9 (21.5%), and DHA (22:6 n-3) (19.0%) (Tveiten et al., 2004). In viable Atlantic halibut eggs, the most abundant fatty acids were 16:0 (17.3%), 18:1 n-9 (9.0%), and DHA 22:6 n-3 (25.9%) (Bruce et al., 1993). In naturally spawned eggs of milkfish, the most abundant fatty acids were 16:0 (32.4%), 18:1 n-9 (15.6%) and DHA (23.5%) (Ako et al., 1994). Other important essential fatty acids found in high quality southern flounder eggs in this study included eicosapentaenoic acid or EPA 20:5 n-3 (3.1%) and arachidonic acid or ARA 20:4 n-6 (1.7%). Differences in egg fatty acid composition may reflect dietary differences among species. Several studies have shown that the quantitative and qualitative lipid content of broodstock diet during gonadogenesis have an influence on

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spawn quality and egg lipid content (Kjørsvik, 1994; Harel et al., 1992; Watanabe et al., 1984a,b; Mourente and Odrioizola, 1990; Watanabe and Kiron, 1995). Egg fatty acid profile in southern flounder probably reflected that of the broodstock diet, which consisted of Atlantic silversides *Menidia menidia*. An interesting study would be to compare wild verses cultured southern flounder embryogenesis fatty acids.

Table 7. Predominant fatty acids in viable eggs of southern flounder and in other species of marine finfish.

	Marine Species			
Fatty acids	Southern flounder[1]	Wolffish[2]	Halibut[3]	Milkfish[4]
14:0	2.1%	1.9%	1.2%	1.3%
15:0	0.9%	0.0%	0.4%	0.0%
16:0	21.5%	13.5%	17.3%	32.4%
17:0	2.9%	0.8%	0.0%	0.0%
18:0	3.7%	3.9%	4.8%	9.0%
18:1 n-9	17.5%	21.5%	9.0%	15.6%
18:1 n-7	4.5%	3.6%	2.2%	0.0%
20:4 n-6	1.7%	1.1%	1.9%	5.6%
20:5 n-3	3.1%	15.8%	11.9%	4.5%
22:5 n-3	6.1%	1.5%	1.4%	0.0%
22:6 n-3	26.1%	19.0%	25.9%	23.5%

[1] *Paralichthys lethostigma* (This study)

[2] *Anarhichas minor* (Tveiten et al., 2004)

[3] *Hippoglossus hippoglossus* (Bruce et al., 1993)

[4] *Chanos chanos* (Ako et al., 1994)

Fatty acid composition and egg quality

In this study, the criteria for evaluating egg quality in southern flounder were similar to those used in halibut, including fertilization success, hatching success, and survival to the first feeding stage (Bruce et al., 1993). No significant differences in total lipid or fatty acid composition between high and low quality southern flounder embryos were observed, in agreement with what Bruce et al. (1993) found in halibut. In southern flounder eggs of high and low quality, the lipid content was 4.4% wet weight of eggs. In wolf-fish, lipid represented 3.9% wet weight of newly developing eggs. In contrast to what was found in southern flounder, wolf-fish larval survival was negatively correlated with %ARA, %EPA, and ARA:DHA and ARA:DHA ratios found in their eggs (Tveiten et al., 2004), suggesting species-specific differences in the influence of lipid composition on egg quality.

Fatty acid composition and embryonic stage

Lipids were not significantly catabolized for energy in southern flounder embryos, as evidenced by a lack of differences in percent lipid, differences in phospholipids or fatty acids among developmental stages between pre-fertilization and hatching. These findings are in agreement with what was found in Eurasian perch *Perca fluviatilis* (Abi-Ayad et al., 2000), plaice *Pleuronectes platessa* (Rainuzzo, 1993), turbot *Scopthalmus maximus* (Planas et al., 1993), winter flounder *Pseudopleuronectes americanus* (Cetta and Capuzzo, 1982) and Senegal sole *Solea senegalensis* (Vazquez et al., 1994). In contrast, significant decreases in lipid content during embryogenesis were reported in a number of species. In cod *Gadus morhua*, egg lipids decreased by 19%

from fertilization to hatching (Frasier et al., 1988). In bluefin tuna *Thunnus thynnus*, egg

lipids in the form of triacylglycerol, decreased 300% (from 420 to 140 mg/g dry egg)

from the early cleavage stage to just before hatching and are important as a main energy

fuel (Takii et al., 1997). In dolphin *Coryphaena hippurus*, catabolism of lipid by

embryos was approximately 2.2 times greater than that by yolksac larvae in terms of

calories consumed per day (Ostrowski and Divakaran, 1991). In red drum *Sciaenops*

ocellatus, neutral lipids contribute up to 98% of energy requirements during

embryogenesis. These results indicate considerable differences among fish species in

lipid and fatty acid utilization during embryogenesis.

In many marine fish species, catabolic rate is much higher during larval

development than during embryogenesis (Hemming and Buddington, 1988). It has been

suggested that most lipids in marine teleost eggs with short incubation times (e.g.

southern flounder) are destined for incorporation into biomembranes rather than for

energy production (Tocher et al., 1985b). In these species, free amino acids (FAA)

appear to be a significant energy source during embryonic development, while fatty acids

from neutral lipids derived from the oil globule are the main metabolic fuel after hatching

(Rønnestad et al., 1994). For example, in turbot *Scopthalmus maximus*, eggs develop

relatively quickly (fertilization to hatch in approximately 4.4 days), and FAAs are used as

an energy source during the egg stage. FAAs are used during the early yolksac stage in

halibut *Hippoglossus hippoglossus*, which have a relatively long embryonic stage (14

days). In lemon sole *Microstomus kitt*, which have an intermediate embryonic period of

6 days, FAAs are important for energy production during both embryonic and early larval

stages (Rønnestad and Fyhn, 1993). In addition to protein synthesis, FAA's participate in

oocyte hydration prior to spawning, hypo-osmotic regulation during early egg development, maintenance of egg buoyancy and energy production during egg and larval development (Fyhn, 1993). Since fatty acids and phospholipids were not significantly consumed during embryogenesis in southern flounder, amino acids in the form of whole protein or free amino acids, and carbohydrate in the form of glycogen were likely used for energy production.

The available data suggests a difference between the embryos of cold-water and warm-water marine finfish species in metabolic energy conversion pathways. The predominance of one source of energy substrate over another is largely attributed to different physiological needs of developing larvae (Ostrowski and Divakaran, 1991). Turbot, a sub-temperate species, which have eggs containing an oil-globule and have a moderate developmental period of four days, tend to conserve PC and use free amino acids and a large lipid reserve (oil droplet comprised mainly of neutral lipids) for energy (Finn et al., 1996). However, in southern flounder there was no measured decrease in percent lipid; also there was an observational lack of size decrease of the oil droplet in the egg during development. Temperate species (e.g. cod and halibut), which lack an oil-globule and have a long developmental period of 10 to 14 days, tend to catabolize PC prior to hatching (Rainuzzo et al., 1992). In cod, a major role of PC was a transporter of neutral lipid and choline from the yolk to the developing embryo and promoting embryonic growth (Frasier et al., 1988). PC, however, was catabolized at a much higher rate in newly- hatched cod larvae than during embryogenesis (Frasier et al., 1988). Tropical species such as dolphin, with rapid developmental periods (1 to 2 days) and relatively high incubation temperatures (>25ºC), have a correspondingly higher energy

demand for cellular metabolism and growth. These conditions may be conducive to promoting the mobilization of more energy dense lipids from the oil droplet and PC for metabolic fuel, while preserving the endogenous amino acid stores for protein synthesis (Ostrowski and Divakaran, 1991).

SUMMARY AND CONCLUSIONS

In summary, there were clear differences in phosphorus composition in viable and non-viable southern flounder eggs. PCr, Pi, ATP and NADP were lower in non-viable eggs indicating a slow down of metabolic activity. PE was higher in unfertilized floating eggs than in unfertilized sinking eggs, but there was no difference in PC. A small PE peak was found in unfertilized eggs. In fertilized eggs, PE increased with development, possibly as a way to conserve DHA and could be related to neuro-visual development. PC did not change significantly and was apparently conserved for later larval development.

ATP levels during embryonic development showed an inverse relationship with pH, probably related to ATP-mediated transport of metabolites. For example, the pH was 7.1 at the blastula stage, 6.3 at the gastrula stage, and stabilized at 6.8 in early embryos, possibly reflecting the accumulation of nitrogenous wastes, followed by active transport of wastes out of the egg.

There were no significant differences in fatty acid composition of fertilized and unfertilized eggs. The most abundant fatty acids separated by class in high quality eggs

included the SFA, palmitic acid, the MFA, oleic acid, and the PUFA, DHA, similar to what was reported in embryos of a number of other species including, turbot, spotted wolf-fish, Atlantic halibut, and dolphin.

There were no significant differences in total lipid or FA composition between high and low quality embryos. Lipids were not significantly catabolized for energy in southern flounder embryos, in contrast to what was found in a number of other marine fish species. Lack of significant decrease of PC and fatty acids during embryogenesis suggests that other components (e.g. free amino acids or carbohydrates) were utilized to meet metabolic needs. Based on these results and those of related studies, we hypothesize that in southern flounder, fatty acids are conserved for utilization after hatching, and that catabolic substrate consumption in embryos starts with glycogen, followed by free amino acids, and then lipid after hatching. This pattern appears to be typical of sub temperate marine fish eggs with an oil globule and relatively rapid developmental period, including southern flounder. More studies involving embryonic free amino acid composition are needed to evaluate this possibility.

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