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● *Original Contribution*

IN VIVO NUCLEAR MAGNETIC RESONANCE IMAGING AND SPECTROSCOPY OF AQUATIC ORGANISMS

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NMR imaging and localized ^{1}H spectroscopy of a variety of aquatic organisms in vivo is described for the first time. The practical consideration of life support, water volume, salinity, and anesthesia are discussed and solutions presented. Such animal studies shape our understanding of physiology, biochemistry, and biology, and provide models of human disease and normal function. These studies also have economic and ecological importance.

Keywords: **MRI; MRS; Aquatic organisms; Fish.**

INTRODUCTION

The study of aquatic organisms shapes our perception of human physiology, biochemistry and biology.[13,15,21,26] Aquatic models of human disease and normal physiologic function are well documented and used extensively in basic and applied research. Studies of sharks[16] and estuarine fishes[6,17,22] provide critical information about membrane transport and osmoregulation. Squid, octopus, aquatic snails, skates, rays, sharks, and bony fishes are all used extensively in neural conduction and function studies.[8,11,24] Invertebrates such as the sea urchin and sand dollar provide valuable information on the cellular and subcellular biochemistry and physiology behind fertilization[25] and cell-cell recognition.[1] Equally important, understanding the biology and ecology of fish, aquatic invertebrates and aquatic plants is seminal to our comprehension of questions related to food production, environmental management, and basic evolution theory.[3,12,18]

The rapid development of nuclear magnetic resonance (NMR) imaging over the past 17 years has created an important tool for examining fundamental clinical and research questions in human medicine.[20] The combination of NMR imaging, and spectroscopy techniques, both localized and unlocalized, provides in vivo biochemical information which is otherwise unobtainable with current technology.[23] In addition, NMR studies of animals, plants, foodstuffs and nonbiological materials[2,14] have provided exciting insights into issues of economic, ecological and intellectual importance.

Studies using combined NMR imaging and spectroscopy promise breakthroughs in areas of biochemistry and physiology where in vivo biochemical information is needed. This includes the study of membrane biology and osmotic regulation.[19] These areas of research where NMR technology has been most useful also depend heavily on the study of aquatic species as models. It is fitting that efforts be made to apply the technology of NMR to the study of aquatic species. In addition, the special abilities of NMR imaging to demonstrate cartilaginous structures and differentiation between tissues of similar radiographic density[20] make NMR exceptionally suited to morphologic studies of elasmobranchs and invertebrates.

This paper presents the successful in vivo NMR imaging and ^{1}H spectroscopy of an aquatic turtle, the yellow-bellied slider (Pseudomys scripta), an elasmobranch, the arabian carpetshark (Chiloscyllium arabicum), a teleost fish, a koi/goldfish cross (Cras-

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sius X), and an aquatic invertebrate, the lion's mane jellyfish (Cyanea capillata). It describes the methods used, and preliminary results obtained, and discusses the utility of NMR techniques for the study of living aquatic organisms including the practical considerations of life support, coil and pulse sequence selection and design.

PRACTICAL CONSIDERATIONS AND METHODS

A primary requisite in any in vivo study is the provision of suitable life support to maintain a physiologically stable subject. Life support requirements are similar for terrestrial and aquatic species,[9] although aquatic species require the additional provision of adequate water volume of appropriate salinity and composition. We developed both closed and open life-support systems to use in NMR imaging studies addressing the following practical considerations.

Water Volume

Oxygen delivery and waste product removal for aquatic species are generally overcome during an experiment by providing adequate water volume to surmount gas solubility and metabolite accumulation limitations. However, in an NMR experiment, particularly one using volume radio frequency (rf) coils, the amount of water surrounding the animal must be minimized to reduce the loading of and the required diameter of the rf coil. Each of these factors is related to the sensitivity of the NMR system. In surface coil experiments, the volume of water surrounding the fish continues to be limiting because of the fixed bore diameter of the magnets.

A flowing multicompartment water system (Fig. 1), avoids the need for large volumes of water around the specimen while preserving an adequate effective volume of water. These support systems can be constructed as open systems allowing ready access to the specimen for manipulation. Alternatively, they can be constructed as closed systems for prolonged studies or those requiring environmental manipulation such as the simulation of depth by increased system pressure.

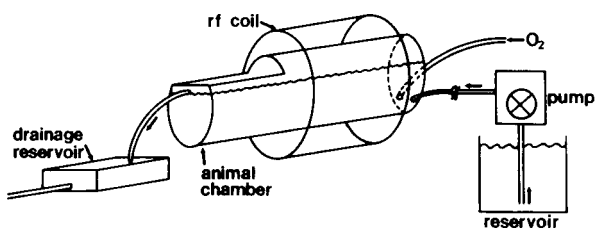

Fig. 1. A diagrammatic representation of the life-support and receiver system used in these studies.

The water volume immediately around the aquatic animals breathing apparatus has the primary impact on the animal's physiology. By constantly exchanging that small volume with water from a larger reservoir, it is possible to simulate much larger water volumes with respect to oxygenation and waste ion elimination. This minimizes the amount of water required to provide protection from dehydration and permit comfortable positioning of the animal. This later factor becomes very important in prolonged experiments. The total volume of water needed in the compartmentalized system depends heavily on the design of the experiment, including the mass of the animal involved and the time course of the study. The degree of water processing incorporated into the multicompartment system is also an important factor. We found that a 10-L system with minimal water processing other than aeration, operating at 220°C, would maintain our small (300–450 g) sharks for 6 h without signs of compromising the animals' baseline responses by measuring its respiratory rate. Larger reservoirs prolong the time the system can be utilized without water changes and smaller animals can be safely maintained in systems longer than larger animals. The advantages of a multicompartment system are purchased at the expense of flow, since the life-maintaining water must be exchanged to ensure the survival of the animal. The flowing water causes flow artifacts in standard imaging sequences which must be eliminated. This problem is addressed in the following section.

Flow

Although continuous flow over the breathing apparatus is preferable for maintaining most aquatic species in a small volume of water, the rate of flow tolerable by the species can become a limiting factor in the design of multicompartment life-support systems. Excessive flow can damage animals not adapted to high-flow living conditions. This is particularly true of animals with relatively sessile life-styles commonly found in relatively still waters.[10] Fortuitously, these animals generally tolerate fluctuations in water quality and oxygenation better than pelagic species.[4] Even very slow flow in a life-support system during NMR imaging causes distortions in the images obtained. Consequently we have developed protocols which image with the flow in the system turned off during image acquisition. Data collection is interrupted at short intervals to allow reoxygenation of the water in the coils and when necessary to temporarily reinstigate

flow. Although it would be feasible to implement imaging pulse sequences that would minimize or eliminate the flow artifacts, turning off the flow has the further advantage of eliminating rf noise from the unscreened pumps which are in close proximity to the magnet (i.e., in the shielded magnet room). An extrapolation of this technique would be to gate acquisition to intermittent flow pumps.

Salinity

The sensitivity of the NMR techniques is dependent upon the salinity of the water needed to maintain the species being studied. Natural seawater is a conductive solution which contains paramagnetic ions. Such a sample heavily loads rf coils, reducing their efficiency. The signal-to-noise differentiation available for animals held in saltwater is consequently much less than that for animals maintained in freshwater. This of course translates into reduced resolution and/or increased imaging times for marine specimens.

The saltwater used for maintaining the marine specimens in our studies was an artificial solution designed to mimic natural sea water (Instant Ocean Synthetic Sea Salts, Aquarium Systems Inc., 8141 Tyler Blvd, Mentor, OH 44060). A series of experiments was performed to determine if the elimination of specific salts which were heavily paramagnetic or a reduction in overall salinity levels would significantly improve the quality factor (Q) which is proportional to the square of the signal to noise ratio.

A phantom was loaded with solutions containing various components of the salt mix used for our marine maintenance water in different combinations and the half height width of the signal taken at 200 MHz in a 6-inch birdcage coil in the earths field and attached to an oscilloscope. This data was compared to the same results with distilled water. From these experiments it appeared that conductivity and not paramagnetic ions contributed the majority of the reduction of Q and the worsening of the signal to noise ratio. There was nearly a 3-fold decrease in Q at 200 MHz for a 26 mg/L solution of NaCl and there was no measurable improvement for solutions down to 4.4 mg/L. Only a 15% increase in Q was achieved with a 2.2 mg/L NaCl solution, which is far too dilute to be useful for maintaining marine specimens. The Q for a 2.5 mg/L $MgCl_2$ solution was identical to that of the NaCl solution. Solutions with seawater concentrations of $CaCl_2$, $NaHCO_3$, and NaBr did not significantly affect the Q of distilled water. Although it is well known that Q is linearly dependent on frequency for a coil of fixed geometry, radius and inductance, which would improve these matters at lower frequencies (i.e., 64 MHz for ^1H in these studies), the required concentration reductions for a significant Q improvement are at least an order of magnitude greater than that which can be tolerated by saltwater aquatic organisms. From this data we decided that the removal of paramagnetic ions from our saltwater mixture was not warranted and that reduction of salinity was not a practical approach to improving the NMR sensitivity in studies of marine specimens. Of course, these problems are eliminated if freshwater species are examined.

Positioning and Immobilization

An important issue in imaging animals in vivo is the requirement to position them without movement for the time the data are being collected. Although the use of multicompartmented life-support systems tends to minimize the volume available for the animals to move in, it does not preclude and may even exacerbate movement of positional adjustment or acoustic startle reactions as the scan is initiated. General anesthesia is therefore necessary in most situations to optimize the data accumulation.

Tricaine methsulfonate (MS222) is a water-soluble anesthetic related to benzocaine which serves well in the safe immobilization of a wide variety of aquatic species. Animals to be anesthetized are usually immersed in a solution containing the anesthetic, most often at a concentration of 0.001 mg/L. When they lose righting reflexes and achieve the appropriate level of immobilization, the specimen can be removed from the solution and placed in an open system life-support apparatus for the duration of the scan. If the data collection is to be prolonged, a dilute solution of MS222 may be necessary in the life-support system to maintain the immobilization. The margin of safety of MS222 is quite wide for most aquatic species if proper oxygenation and water quality are maintained. Patients recover when returned to water not containing the drug. The length of the recovery period is directly related to the length of the immobilization period.

In closed system life-support apparatus where direct access to the animal is not feasible, anesthesia can be accomplished by flushing the holding compartment with anesthetic for induction. Reversal is achieved by reinstating the flow of unadulterated water. To accomplish this procedure safely, it is important to know precisely the flow rate of the system, the volume of the holding chamber, the delay to delivery in the holding chamber from reservoirs, and the effective flushing time of the system. We measure these characteristics in newly constructed life-support systems before using them to hold animals by introducing a small amount of concentrated phenol red dye into the anesthetic

reservoir. Then we measure the time to its appearance and disappearance from the holding chamber.

Aquatic turtles can be held out of water for extended periods without harm. This allows the use of manual restraint for most studies. This is readily accomplished by taping the turtle to a block of foam rubber just slightly narrower than the plastron width and higher than the turtle's leg length. Studies of the heart of turtles are facilitated by anesthesia with ketamine which has the benefit of depressing the heart rate of the turtle, reducing the motion artifact from the beating heart.

Animals

The teleost fish selected for these studies were hybrid koi/goldfish crosses (Crassius X). They were maintained on a diet of trout chow in a 100 gallon aquarium with biological filtration. The elasmobranchs used in this study were young Arabian carpet sharks (Chilosyllium arabicum), maintained in a 300-gallon closed artificial seawater system with mechanical filtration. They were fed a diet of chopped fish and invertebrates. The aquatic invertebrates used in these experiments were lion's mane jellyfish (Cyanea capillata) collected from the Baltimore harbor using hand nets. They were not maintained in the laboratory for more than 24 hr. The gravid yellow-bellied slider turtle (Pseudomys scripta) imaged was a display specimen from a local aquarium. It was kept in a freshwater aquarium and fed a variety of chopped fish and invertebrates.

Imaging and Spectroscopy Protocol

All NMR data was obtained on a 1.5 Tesla whole body General Electric Signa MRI machine. These studies were performed using a commercial General Electric 17-cm diameter knee coil. This coil is constructed in two halves. The top half is removable and allows fast and easy access to the subject. This is an important consideration during anesthesia. In all studies, conventional two-dimensional Fourier transform spin-echo[5] and inversion-recovery imaging pulse sequences were used. The localized ^{1}H spectra were obtained using the STEAM technique.[7] In all cases a TE of 30 msec was used.

RESULTS

Figures 2–6 show example images of the goldfish, sharks, jellyfish, and turtles used in these studies. All animals were anesthetized as previously described. Note especially the absence of motion artifacts in these images.

Figures 7 and 8 show examples of ^{1}H spectra obtained on a turtle and a shark. No suppression or editing methods were used. We expect to incorporate

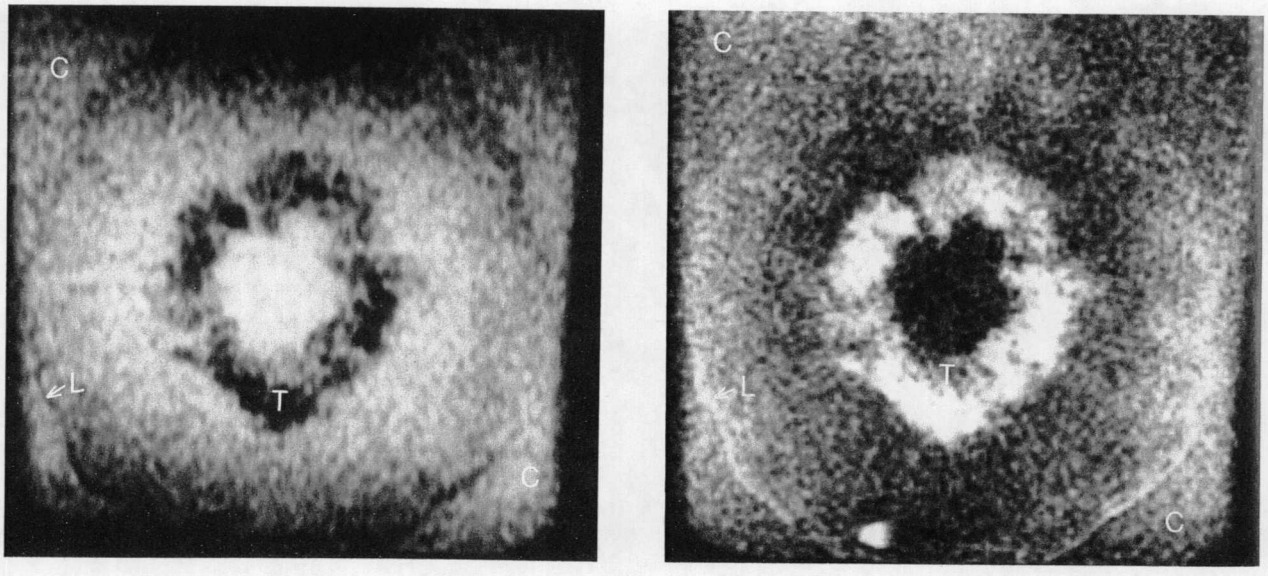

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Fig. 2. In vivo MR images of a common jellyfish (Cyanea capillata) anesthetized with MS222. (a) Spin-echo image (TR = 3000 msec, TE = 20 msec, resolution = 0.5 × 0.5 × 5 mm, imaging time = 25.6 min). The outer membrane or limbus (L), tentacles and tentacle support apparatus (T) and the surrounding water in the container (C) are indicated. (b) Inversion-recovery image (TI = 1200 msec, TR = 4000 msec, resolution = 0.5 × 1 × 5 mm, imaging time = 11 min).

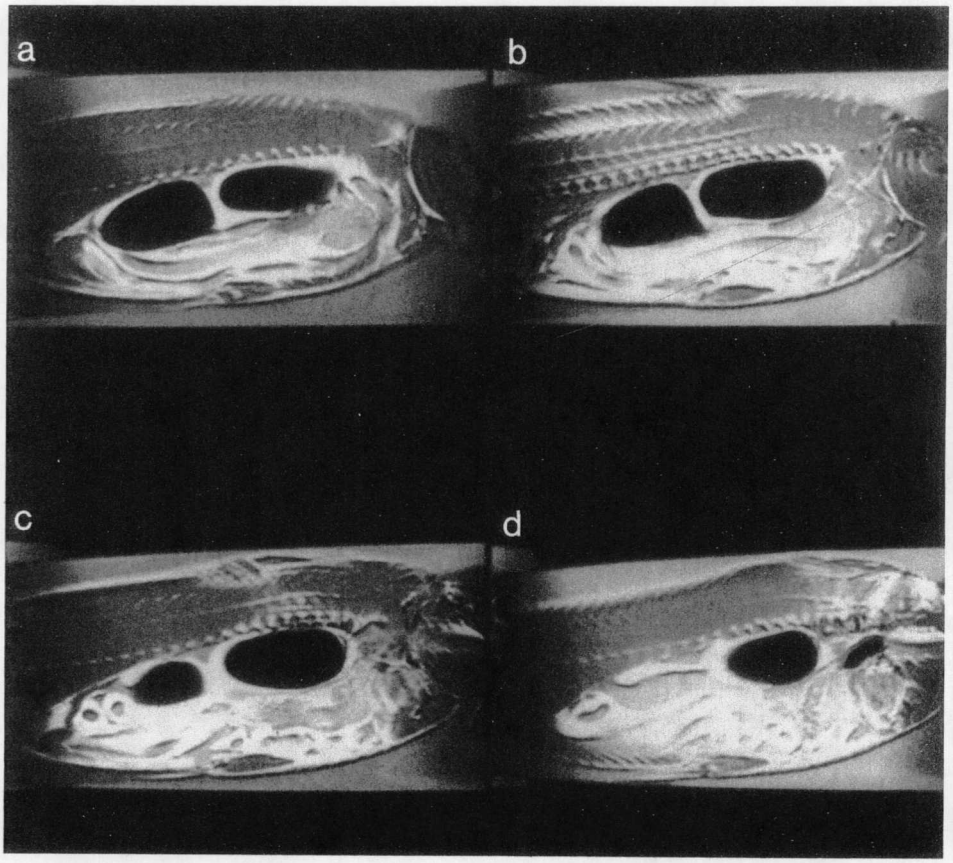

Fig. 3. (a–d) Four consecutive sagittal in vivo MR images (1 mm between slices) of a goldfish/koi hybrid (Crassius X) anesthetized with MS222 (TR = 100 msec, TE = 20 msec, resolution = 0.6 × 0.6 × 3 mm, imaging time = 8.5 min). The two large dark areas are the swimbladder, directly ventral to the spine.

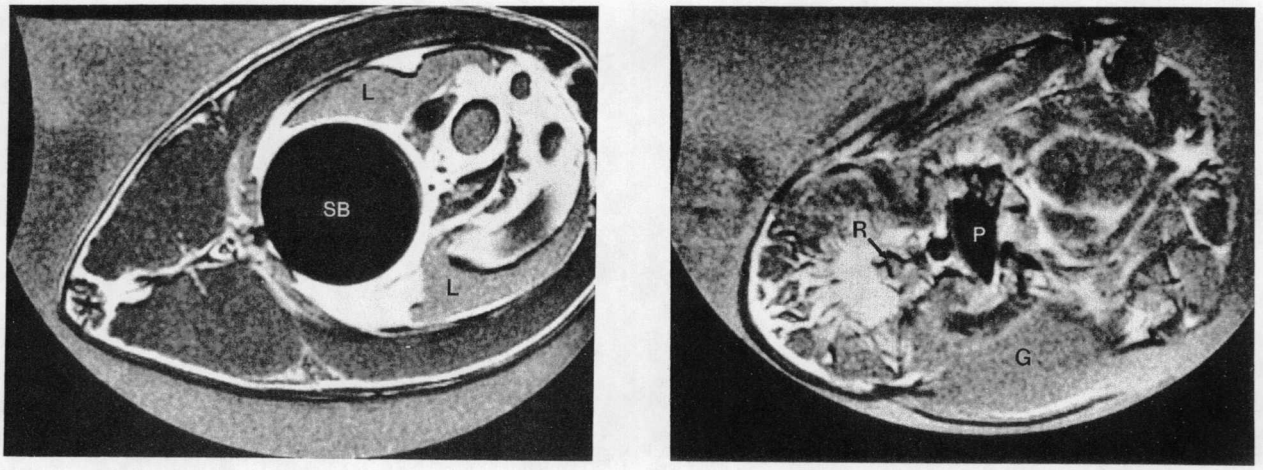

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Fig. 4. Two axial images through the same goldfish as in Fig. 3 (TR = 1500 msec, TE = 20 msec, resolution = 0.3 × 0.3 × 3 mm, imaging time = 12.8 min). (a) A section through the anterior chamber of the swimbladder (SB) and left and right lobes of the liver (L). (b) A section close to the head of the goldfish showing the posterior rhombencephalon (P), oral pharynx (P), and gill (G).

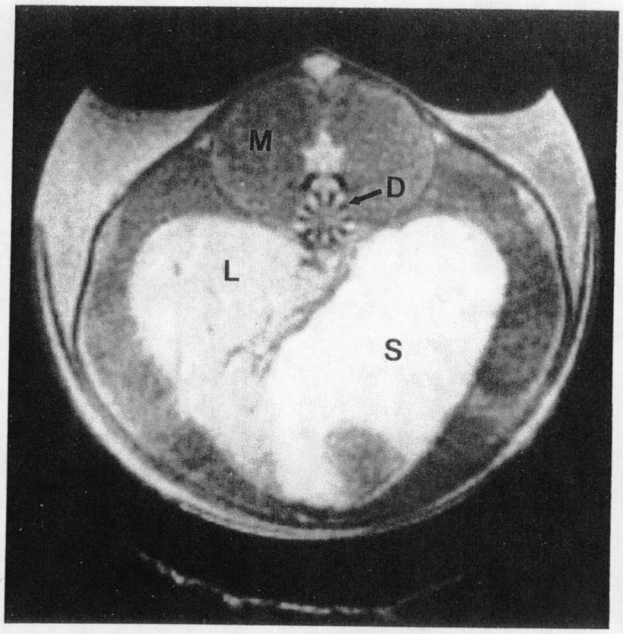


Fig. 5. An in vivo axial spin-echo image (TR = 1500 msec, TE = 30 msec, resolution = 0.3 × 0.3 × 5 mm, imaging time = 6.4 min) through an Arabian carpetshark (Chiloscyllium arabicum) anesthetized with MS222 showing the liver (L), the stomach (S), vertebral disc (D), and lumbar musculature (M).

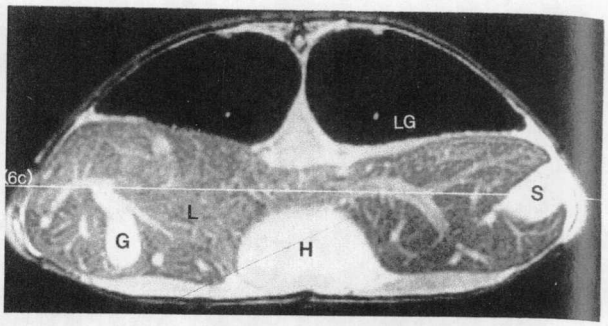
water and lipid-suppression methods when shielded gradients are installed on the imaging machine.

CONCLUSION

The feasibility of in vivo MR imaging of aquatic organisms has been demonstrated. Conventional imaging and spectroscopic techniques may be used in conjunction with a suitably designed aquatic life support system. The ^{1}H spectra obtained in these studies can be used to examine the effects of diet or toxicity on hepatic lipid accumulation in sharks or embryonic nutrition in the large eggs (1–2 cm in diameter) of the turtles.

The noninvasive in vivo examination of aquatic organisms has broad application in the study of economic, environmental, and ecological concerns. Clinical applications in economically important species include the evaluation of disease processes for diagnosis and treatment. Valuable information concerning the physiological and biochemical responses of aquatic organisms to disease or environmental factors can be obtained. The noninvasive nature of the procedures makes their use in the study of endangered or physically delicate species potentially valuable.

Anesthesia is essential for eliminating motion artifacts and damage to those subjects which are other-



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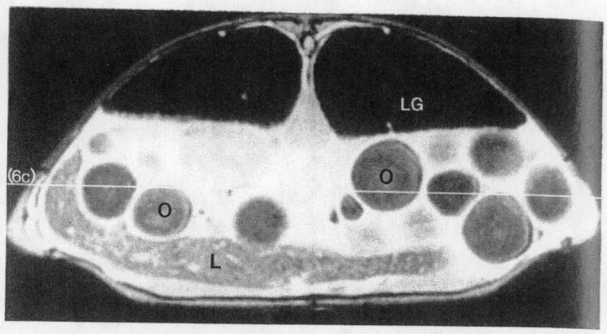

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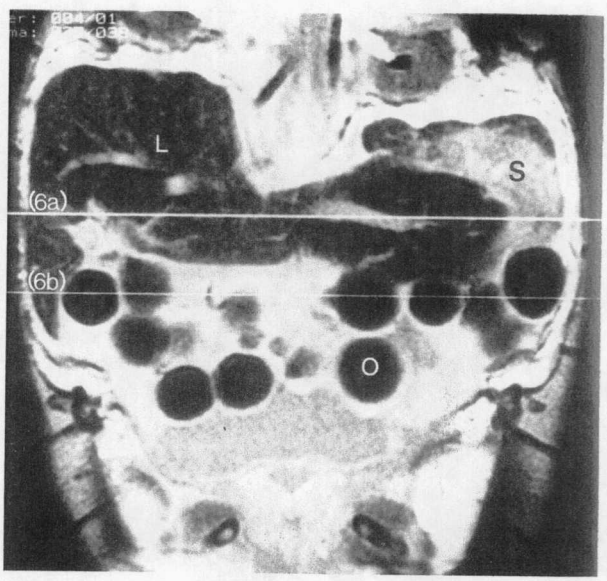

c

Fig. 6. In vivo MR images of a gravid yellow-eared slider (Pseudomys scripta) anesthetized with ketamine HCl. (a) Axial spin-echo image through the level of the liver showing the gallbladder (GB), heart (H), lungs (LG), and cardia of the stomach (S). (TR = 2000 msec, TE = 20 msec, resolution = 0.45 × 0.45 × 3 mm, imaging time = 8.5 min). The motion artifact from the heart is minimal because of the very slow heart rate of anesthetized turtles (3–10 beats per min). (b) Axial spin-echo image posterior to Fig. 6(a) showing developing ova (O) in addition to the liver (L) and lungs (LG). (c) A coronal spin-echo image at the level marked on images 6(a) and 6(b) showing developing ova (O), the liver (L) and the cardia of the stomach (S). The lines on this image mark the locations of images 6(a) and 6(b).

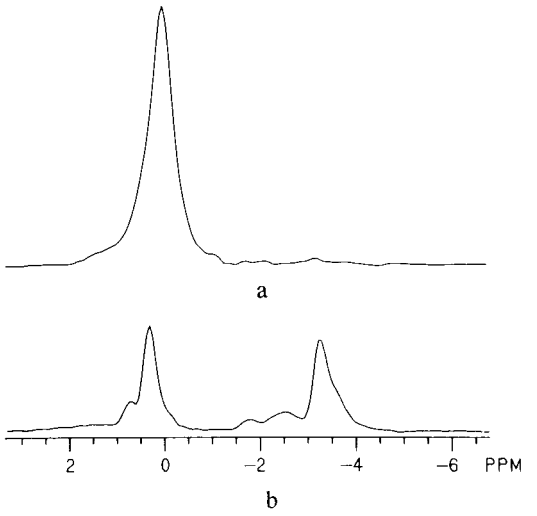

Fig. 7. Spatially localized ^1H spectra from a gravid yellow-eared slider. The localized volume was a 1-cm cube. (TR = 3 sec, 16 averages). The spectra are localized to (a) muscle and (b) an ovum.

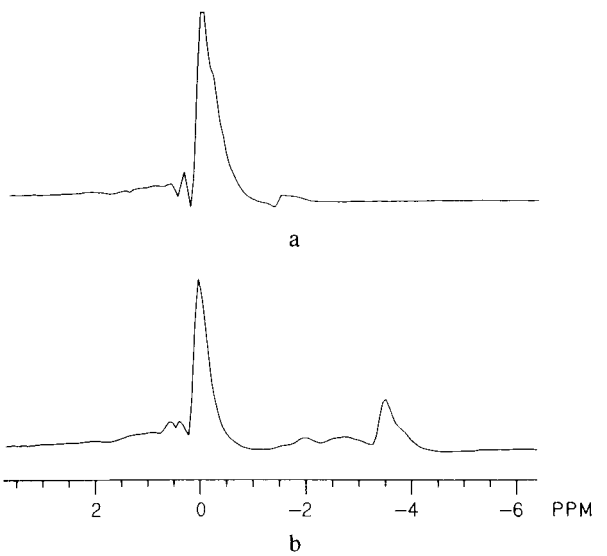

Fig. 8. Spatially localized ^1H spectra from a shark. The localized volume was a 6-mm cube. (TR = 3 sec, 16 averages). The spectra are localized to (a) muscle and (b) the liver.

wise startled into escape responses by the acoustic gradient vibrations during the image acquisition procedure. Anesthesia of the fish and the invertebrate jellyfish can be accomplished using MS222 as an immersion drug in low concentrations. Imaging of turtles is facilitated by the use of ketamine HCl anesthesia to reduce motion but high quality images and spectra can be obtained from properly restrained unanesthetized turtles. In vivo MR imaging and spectroscopy promise to be practical and effective tools in the study of aquatic species.

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