Heavy Water (D₂O) Alters the Sodium Channel Gating Current in Squid Giant Axons
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When nerve axon membranes are abruptly depolarized, a small outward displacement current precedes the sodium current that underlies the propagated nerve impulse. This displacement current is asymmetric; it does not appear in a symmetrical hyperpolarization. It was named “gating current” by earlier workers because of its association with the opening of the activation gates of the sodium channels. The gating currents were isolated by replacing permeant ions with impermeant ones, thus reducing ionic currents, subtracting away symmetrical currents and, in most cases, blocking the ionic sodium current with tetrodotoxin (1, 2).

Replacing the H₂O in solutions with D₂O slows many chemical and biological reactions including the squid axon action potential (3), ionic currents (4), and the sodium pump (5). However, Meves (6) reported that D₂O had no significant effect on the asymmetry currents of squid axons. This result was confirmed in Myxicola (7) and crayfish (8) axons. Most measurements were made at voltages less than +20 mV, which would not be expected to open all of the channels, and in the presence of tetrodotoxin. Both the ionic and gating currents can be recorded if the experiments are carried out in solutions with low sodium content (9). When this is done, D₂O can be seen to reduce the amplitude of the gating currents at more positive potentials.

Segments of squid axons were bathed in an artificial seawater containing 44 mM NaCl, 396 mM tetramethylammonium (TMA) chloride, and 2 mM TMA Hepes, pH 7.4; and the internal Cs perfusion fluid contained 150 mM Cs glutamate, 50 mM CsF, 750 mM sucrose, and 40 mM Cs Hepes, pH 7.4. Solutions were made up with either H₂O or 99.8% D₂O. The axons were voltage-clamped at a −70 mV holding potential. Gating currents were recorded with a p/8 protocol, as follows: the holding potential was shifted to −140 mV, and 8 small “subtraction” pulses, ⅛ the amplitude of the test pulse, were applied and their currents summed. Then the potential was shifted back to −70 mV, and a single test pulse was applied. This procedure was repeated every two seconds. Currents were filtered at 40 kHz and sampled at 100 kHz. The records presented are the difference between the test current and the summed subtraction currents averaged over 64 cycles. Experiments were performed at 3°–4°C.

The figure shows records made with pulses to +25 and +50 mV. The effect of D₂O (filled symbols) is to reduce the initial outward gating current by about 30%, to increase the time to peak inward current to about 1.4 times its value in H₂O, and to slow the decline of inward current associated with inactivation of the sodium channels. The sodium conductance was reduced by about 35%. The changes in ionic currents are similar to those previously described (3, 6, 7). In 56 measurements at 0 to +75 mV on 11 axons in the absence of tetrodotoxin, D₂O reduced the peak of the

Figure 1. D₂O alters gating and ionic currents. Open symbols are currents in H₂O-based solutions; filled symbols indicate D₂O-based solutions. The records are for a steps from a −70 mV holding potential. The upper records are to +25 mV; the lower, to +50 mV. The records on the right are shown at the expanded timebase.
initial outward gating current to 0.70 ± 0.02 times its value in \(H_2O\).

The simplest interpretation of these results is that \(D_2O\) slowed the rate of the conformational change by 30%, thus reducing the amplitude of the gating current and increasing the time required to open the channels. This could occur by changing the channels or changing the environment in which they operate. The viscosity of \(D_2O\) is larger than that of \(H_2O\), and in fact, the reduction of gating and the slowing of ionic currents described above are qualitatively similar to those seen in solutions with a viscosity that has been increased with non-electrolytes (10). On the other hand, the \(D_2O\) effect seems larger than predicted by viscosity alone. Perhaps \(D_2O\) alters the gating machinery. About 40% of the amide protons of the *Streptomyces lividans* K+ channel exchange within 3 minutes of \(D_2O\) exposure accompanied by subtle structural changes (11). To test between these two possibilities, currents were recorded during the transition from 0 mM sodium \(H_2O\) seawater into 44 mM sodium \(D_2O\) seawater. In the 0 mM Na \(H_2O\) solution, there was no inward sodium current. By 90 s after beginning the switch into the 44 mM Na \(D_2O\) solution, the inward current appeared—but in the \(H_2O\) pattern, similar to the open symbols in Figure 1. Over the next 3 min the current pattern switched to the \(D_2O\) pattern, similar to the filled symbols. This suggests that the \(D_2O\) effect involves changes in channel structure.

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**Voltage Gating Properties of Channels Formed by a Skate Retinal Connexin**

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Gap junctions provide pathways for electrical and chemical communication between networks of coupled cells. They act as simple electrical synapses, and also synchronize and regulate a broad range of cellular activities. The structural proteins constituting gap-junctional channels in vertebrates are the connexins, members of a multigene family that exhibit a common topology: four transmembrane domains separating two well-conserved extracellular loops and three cytoplasmic domains. Six connexin polypeptides oligomerize to form a membrane hemichannel or connexon, clusters of which join with the connexons of adjacent cells to create the gap junction. Once assembled, the gap-junctional channel consists of an aqueous pore that allows the cell-to-cell diffusion of ions, second-messenger molecules, and small metabolites. Different cell types contain connexins that are unique to their special needs, and variations in the molecular structure of the individual connexins determine the gating properties, voltage dependence and cellular interactions of their gap-junctional channels (1).

The vertebrate retina is a useful model with which to study the diversity of electrical coupling in nervous tissue. Diverse experimental approaches have shown that virtually every class of retinal neuron and glial cell makes gap junctions with neighboring cells of similar type, and in some cases with cells of another type (2, 3). Moreover, coupling between different cell types appears to be mediated by gap junctions that exhibit asymmetric dye transfer, as well as distinct pharmacological properties (4, 5). Although there is abundant evidence that the electrical synapses formed by gap junctions affect every aspect of retinal function, relatively little is known about the connexins mediating these effects. The situation has been changed by the identification of a distinct subgroup (γ) of the connexin family that shows a pattern of expression restricted to the retina and the central nervous system (6–11). The first member to have been discovered, Cx35, was cloned from a skate retinal cDNA library (6), and some of its functional characteristics were examined in the *Xenopus* oocyte expression system (12). In the present study, we have extended these observations to analyze more fully the voltage sensitivity and kinetics of the gap junctions formed by Cx35 in paired oocytes. In addition, we have investigated the properties of the non-junctional hemichannels formed in single oocytes, and have compared the effects of quinine on the kinetics of the tail currents evoked at the termination of voltage pulses.

The procedures for preparing cRNA, and its analysis in *Xenopus* oocytes have been described previously (12). Oocytes were isolated by enzymatic digestion and injected with either an antisense oligonucleotide (3 ng/cell) to suppress the endogenous *Xenopus*
Figure 1. Voltage gating of channels in Xenopus oocytes expressing skate Cx35. (A) Gap junctional currents ($I_j$) were elicited by transjunctional voltage ($V_j$) steps, 4 s in duration, applied in ±20 mV increments from a holding potential of -40 mV. (B) Plotting steady state junctional conductance ($G_{jss}$ normalized to the values measured at ±20 mV) vs. $V_j$ shows that, even at the extremes of $\Delta V_j$ (±120 mV), the residual conductance is equal to about half the initial value; data are the means (±SD) of 4 cell pairs. Curves drawn through the data were derived from the Boltzmann equation in which the parameters for positive values of $V_j$ were $A = 0.06$; $V_o = 95$; $G_{max} = 1$; and $G_{min} = 0.40$. For negative values of $V_j$, the corresponding values were 0.05,
Cx38 (13), or a combination of antisense (as above) plus Cx35 RNA (5 ng/cell). To study intercellular channels, oocytes were stripped of their vitelline membranes and brought into contact at their vegetal poles for 48 h before electrophysiological analysis. This step was omitted to explore hemichannel activity, which was recorded 48–72 h after RNA injection.

Intercellular communication was quantified by dual cell voltage clamp (12, 14). To determine the voltage-gating properties of the intercellular channels, transjunctional potentials (Vj) of opposite polarity were generated by hyperpolarizing or depolarizing one cell in 20 mV steps (over a range of ±120 mV), while clamping the second cell at −40 mV. Currents were measured 4 s after the onset of the voltage pulse, at which time they approached steady state (Ijss), and the macroscopic conductance (Gjss) was calculated by dividing Ijss by Vj. Gjss was then normalized to the values determined at ±20 mV, and plotted against Vj. Data describing the relationship of Gjss as a function of Vj were fit to a Boltzmann relation equation yielding V o values of the initial value, was still present at the extremes of the voltage (±120 mV), and the current decay approached steady-state levels by the second cell at 60 mV. Currents were measured 4 s after the onset of the voltage pulse, at which time they approached steady state (Ijss), and the macroscopic conductance (Gjss) was calculated by dividing Ijss by Vj. Gjss was then normalized to the values determined at ±20 mV, and plotted against Vj. Data describing the relationship of Gjss as a function of Vj were fit to a Boltzmann relation (14) of the form: 

\[ G_{jss} = \frac{G_{jmax} - G_{jmin}}{1 + \exp\left[\frac{V_j - V_o}{A}\right]} + G_{jmin}, \]

where Gjss is the steady-state junctional conductance, Gjmax (normalized to unity) is the maximum conductance, Gjmin is the residual conductance at large values of Vj, and Vm is the transjunctional voltage at which Gjss = (Gjmax − Gjmin)/2. The constant A (=nq/kt) represents the voltage sensitivity in terms of gating charge as the equivalent number (n) of electron charges (q) moving through the membrane, k is the Boltzmann constant, and T is the absolute temperature. The time constants (τ) of voltage-dependent transitions of junctional conductance were calculated using data-fitting functions in Microcal Origin. To characterize the hemichannel activity of connexons, non-junctional current recordings were obtained from single oocytes with a two-electrode voltage-clamp procedure. Cells were clamped at −40 mV, and whole cell currents recorded in response to depolarizing voltage steps (from −20 to +80 mV in 20-mV intervals) imposed for 5 s.

In a previous study (12), we restricted our observations of voltage gating to transjunctional potentials of ±80 mV and did not quantify voltage dependence. In the current study, we have extended this analysis to much larger values of Vm and have quantified the degree and kinetics of voltage gating. Figure 1A illustrates a family of gap-junctional currents elicited at transjunctional voltage steps up to ±120 mV in oocyte pairs expressing Cx35. Voltage gating occurred symmetrically at the higher values of Vj (80–120 mV), and the current decay approached steady-state levels by the end of the imposed step. Quantitative analysis (Fig. 1B) indicated that a large residual conductance, equivalent to approximately half of the initial value, was still present at the extremes of the voltage range tested. Fitting the Gj vs. Vj relationship to the Boltzmann equation yielded V o values > 80 mV (see figure legend) confirming the relatively weak Vj gating described earlier. Thus, voltage is not likely to be a primary modulator of Cx35-mediated intercellular communication in retinal neurons.

The voltage gating characteristics of Cx35 were further explored by analyzing the kinetics of channel closure for values of Vj ≥ 80 mV, i.e., sufficient to consistently induce current decay. Figure 1C illustrates results obtained from one cell pair in response to a transjunctional voltage step of +100 mV. The time-dependent decline in Ij was well fit by a single exponential function with a time constant (τ) of 0.448 s. Interestingly, the mean values of τ, obtained both for different values of Vj, as well as for positive and negative voltage steps, hovered about 0.4 s and showed no significant change as a function of either the polarity or the magnitude of Vj (Fig. 1D). These data are in sharp contrast to kinetic analyses of many other connexins, where τ values decreased with increasing driving force (15, 16). This feature is shared by another γ connexin, mouse Cx36 (data not shown), and illustrates further the unique properties of this subgroup.

The ability of connexins to form hemichannels in Xenopus oocytes, a property reminiscent of membrane currents observed in some retinal neurons (17, 18), prompted us to investigate the kinetics of Cx35 hemichannel closure by analyzing tail currents. As we showed previously, quinine-sensitive hemichannel currents can be recorded from oocytes expressing skate Cx35 (12). This is confirmed in Figure 1E, which shows the increase in the outward (non-junctional) current recorded from a single oocyte in response to depolarizing voltage increments ≥ 40 mV, and the current enhancement produced by the addition of 100 μM quinine to the normal bath solution. To determine whether quinine exerted an effect on the gating properties of the hemichannels, we measured the kinetics of the tail currents recorded at the termination of the voltage step. Figure 1F shows that the mean of the time constants of the single exponential decay functions describing the data for the return of Vm to −40 mV from values of +40 to +80 mV were unaffected by quinine. However, the hemichannel time constants are not directly comparable to the intercellular channel τ values, as the ionic strength of control bath solution is greatly reduced in comparison to ooplasm, and K + is replaced by Na + as the principal cation. Further studies are required to determine the precise relationship between hemichannel and intercellular channel gating, and to clarify the mechanism whereby quinine markedly increases Cx35 mediated hemichannel currents.

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Hydrogen Ion Fluxes from Isolated Retinal Horizontal Cells: Modulation by Glutamate

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Retinal horizontal cells are second order neurons that receive direct input from photoreceptors. These cells are believed to play a crucial role in the formation of the surround aspect of the classic center-surround receptive fields of visual neurons. Debate still persists as to the molecular mechanisms used by horizontal cells to establish the surround portion of these receptive fields. One hypothesis, promulgated recently by Kamermans and colleagues (1), suggests that horizontal cells may exert their lateral inhibitory actions by modulating the calcium flux into the synaptic terminals of photoreceptors, thus altering the release of the photoreceptor neurotransmitter. Hydrogen ions are among several agents that should be modified by glutamate, the neurotransmitter believed to be released by vertebrate photoreceptors (7). The pH-selective electrodes were used in a self-referencing mode (8), which greatly enhances their signal sensitivity and stability, eliminating much of the electrical noise and drift inherent in such electrodes. In this format, the electrode is first placed just adjacent to the membrane of the cell, and a reading taken; the electrode is then moved a set distance away (typically 30 μm), and a second reading taken. The difference between the voltage readings at the two positions reflects differences in the free hydrogen ions from horizontal cells is indeed a key factor in the creation of the surround portion of retinal receptive fields, then such a flux should be modified by glutamate, the neurotransmitter believed to be released by vertebrate photoreceptors (7).

The pH-selective electrodes were used in a self-referencing mode (8), which greatly enhances their signal sensitivity and stability, eliminating much of the electrical noise and drift inherent in such electrodes. In this format, the electrode is first placed just adjacent to the membrane of the cell, and a reading taken; the electrode is then moved a set distance away (typically 30 μm), and a second reading taken. The difference between the voltage readings at the two positions reflects differences in the free hydrogen activity at the two locations. This method allowed us to measure the small hydrogen ion fluxes that would otherwise have been lost in the noise of the recordings.

pH selective electrodes were prepared by pulling thin-walled glass capillary tubing (o.d. 1.5 mm) to a tip diameter of 2–4 μm. The pipettes were silanized and back-filled with 100 mM potassium chloride, and the fluid was forced to the tip of the pipette by air pressure applied to the back of the pipette from a syringe. The pipette tip was then filled with a pH-sensitive resin (hydrogen ionophore 1-Cocktail B, Fluka Chemica); the tip was placed in contact with a source pipette containing the resin, and about 50 μm

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of the resin was then drawn up by suction on the back of the pipette. The resin employed here has a particularly high selectivity for hydrogen ions, and is reported to be more than 10^9 times more sensitive to hydrogen ions than to either sodium or potassium ions (9). Isolated retinal horizontal cells were obtained by enzymatic dissociation of the retina of the skate (Raja erinacea or R. ocellata) as described in Malchow et al. (10). Briefly, the animals were chilled in ice, cervically transected, and double pithed. The eyes were removed, and the retinas were isolated and placed for 45 min under gentle agitation into a skate-modified L-15 culture medium containing 2 mg/ml papain and 1 mg/ml cysteine. The retinas were then rinsed 8 times in media lacking papain and cysteine, and then mechanically agitated through a 3-ml graduated glass pipette. Single drops of this cellular suspension were placed in 35-mm plastic culture dishes that had previously been coated with 1% protamine sulfate and 0.1% concanavalin A. Cells were stored at 14°C for up to 4 days before use. Recordings were made in a skate Ringer’s solution containing 2 mM of the pH buffer HEPES and no added bicarbonate. A 5 mM glutamate stock solution was prepared in skate Ringer and adjusted to pH 7.6 with 1 M NaOH. Glutamate was applied by adding 1 ml of the 5 mM glutamate solution to 4 ml of Ringer already present in the culture dish, resulting in a final concentration of 1 mM glutamate.

Under these conditions, a steady differential signal was obtained from horizontal cells indicative of a higher concentration of hydrogen ions near the membranes of the cells. The size of this signal decreased as the concentration of the pH buffer HEPES was increased, consistent with the hypothesis that the signal detected indeed reflected hydrogen ions. Moreover, as shown in Figure 1, the application of 1 mM glutamate resulted in a marked decrease in the size of the differential signal. A differential signal of approximately 100 μV was initially recorded from this cell. The actual proton flux represented by this differential voltage can be calculated using an equation derived by D. M. Porterfield [in prep.; see also (12)] as follows:

\[ J = -D(\Delta[H^+] + [\text{Buffer}] \ast 0.25\Delta[H^+] \ast K_a^{-1}) \ast \Delta r^{-1} \]

Where J is the flux, D is the diffusion coefficient for hydrogen ions, \(\Delta[H^+]\) the change in hydrogen ion activity between the two poles of measurement, [Buffer] is the buffer concentration expressed in moles per cm^{-3}, \(K_a\) is the pK_a of the buffer expressed in cm^{-3}, and \(\Delta r\) is the distance in cm between the two measuring positions of the probe. Taking into account a small loss of the signal within the electronics of the amplification system (8), under our experimental conditions the 100 μV signal we observe is then estimated to be indicative of a proton flux of \(\sim 75 \text{ pM cm}^{-2} \text{ s}^{-1}\). In 6 cells studied in this fashion, 1 mM glutamate reliably reduced the differential signal by an average of 60%.

We thus conclude that glutamate, the presumed neurotransmitter from vertebrate photoreceptors, can indeed alter the flux of hydrogen ions from horizontal cells. In this context, it is interesting to note that glutamate has previously been reported to promote an acidification of the internal milieu of catfish retinal horizontal cells as measured using the pH-indicator dye BCECF (11). We hypothesize that glutamate may shut down the transport of hydrogen ions from horizontal cells, thus trapping hydrogen ions in the interior of the cell. This would account for the increased intracellular acidity and the alkalinization of the extracellular milieu that we have observed. The alteration in extracellular pH induced by glutamate may be important in modifying signaling within the outer plexiform layer of the retina. Indeed, extracellular alkalinizations induced by neuronal activity occur in several other regions of the nervous system (reviewed by Chesler (13)), and excitatory amino acid receptors have been implicated in the generation of these phenomena. Thus, modulation of extracellular pH within the CNS by glutamate may be a common means by which synaptic activity is altered. Future experiments are planned in which specific pharmacological agents will be used to determine which transporter or transporters may be involved in the glutamate-induced changes in extracellular hydrogen ion concentrations.

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**Figure 1.** Effects of glutamate on the hydrogen ion flux recorded from a single isolated retinal horizontal cell. The differential voltage recorded from a pH-selective electrode is plotted as a function of time. Before the application of glutamate, a differential signal indicative of a higher concentration of hydrogen ions near the membrane of the cell is observed. At the arrow, glutamate was added such that the final concentration in the dish was 1 mM. A marked decrease in the differential signal recorded by the pH-selective electrode was observed.

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Odor-induced Oscillatory Activity in Drosophila CNS

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In mammals and the fruit fly, the vast array of odors in the environment is discriminated by a large number of receptor molecules (1, 2, 3). Individual olfactory sensory neurons express only one of the many receptor genes (1, 2, 3). Neurons expressing the same receptor gene project to the same glomerulus (4, 5, 6), providing the anatomical evidence for a spatial coding mechanism. Electrophysiological recordings from olfactory neurons suggest that the temporal pattern of their responses can also convey information about odor quality (7). Odor-induced oscillatory activity, an indication of synchrony, has been observed in phylogenetically different species, including molluscs, insects, and mammals (7, 8, 9, 10, 11, 12).

The adult Drosophila antennal lobe, organized in spheroidal subcompartments termed glomeruli, receives about 1200 olfactory afferents from the antenna and 120 afferent fibers from the maxillary palp (13). Although the fly and mammals share the similarity that receptor neurons expressing the same receptor gene project to one or two glomeruli in a stereotypic manner (4, 5, 6), there are only 60 receptor genes and 43 glomeruli in Drosophila, in contrast to the 1000 receptor genes and 1800 glomeruli within the olfactory bulb of mammals (1, 2, 3). The lower complexity in anatomy and the rich behavioral repertoire in Drosophila makes it an attractive system with which to study olfaction. Moreover, sophisticated genetic tools and behavioral mutants can now also be used to study the olfactory system in Drosophila. Nevertheless, understanding mechanisms of odor discrimination in the CNS of the fly has been difficult due to a lack of physiological tools for functional studies. Odor-induced oscillations have been observed in several insect species, including the locust, cockroach, honeybee, bumblebee, and wasp (7). Local field potential (LFP) recordings show odor-induced oscillation at ~10 Hz which typically lasts for the duration of odor stimulation. I have investigated this phenomenon in the Drosophila CNS. LFPs were recorded with glass electrodes (tip, 5 μm) that were filled with Drosophila HL3 saline and

![Figure 1](image)

Figure 1. Local field potential recordings of odor-induced oscillation in the CNS of Drosophila. The left panel shows five sequential responses to peppermint stimulation recorded from the same preparation. Responses to amyl acetate from the same preparation are shown in the middle panel. Averaged power spectrum density from the five trials is shown in the right panel. The LFP response to peppermint appears to have a higher density at 2 Hz than the response to amyl acetate.
positioned with a motorized manipulator (MP285, Sutter). A patch clamp amplifier (EPC 7, Heka) was used, and the signal was filtered (band pass at 0.1 to 20 Hz) with a signal conditioner (CyberAmp, Axon Instruments) and recorded with software (AxoScope, Axon Instruments) run on a PC. Adult flies (less than a week after eclosion) were lightly anesthetized with CO₂ and decapitated. The heads were immobilized with wax on a microscope slide with the antennae pointing upward. A small opening was made on the dorsal cuticle for the extracellular recording.

Figure 1 shows LFP recordings from the CNS of the Canton-S wild-type fly that reveal an odor-induced oscillation. This phenomenon was confirmed in 6 preparations. A power spectrum analysis indicates that the major frequency components are less than 4 Hz (Fig. 1). This LFP oscillation signal appears to be sensitive to the position of the electrode, and the coordinates taken from the manipulator suggest that the recordings may have originated in the antennal lobe. Future experiments with GFP-labeled antennal lobe may help in identifying the sources of the oscillatory activity. The patterns of oscillation in response to the same odor appear to be roughly similar in sequential recordings from the same animal. The LFP patterns generated in response to peppermint (from McCormick) and amyl acetate (from Sigma) were distinguishable by eye. Moreover, the power spectrum analysis indicates that peppermint generates slightly more high frequency components.

This is the first LFP recording from the Drosophila CNS. The preliminary results presented here show that odor-induced oscillation occurs in Drosophila; this finding suggests that a temporal coding mechanism may be employed by the fly, and that the power of genetics may be applied in the future to decipher the physiological significance of the odor-induced oscillation.

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Circadian Rhythms in the Receptive Fields of the Limulus Lateral Eye

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Hartline found that in the frog “a given optic nerve fiber responds to light only if a particular region of the retina receives illumination.” He called the region the receptive field of that fiber (1). Continuing Hartline’s study of the frog retina, H. B. Barlow detected an inhibitory influence on the excitatory region of the receptive field (2). In the lateral eye of the horseshoe crab Limulus polyphemus, the receptive fields of single ommatidia have both excitatory centers and inhibitory surrounds. The field of view of a single ommatidium defines the narrow excitatory center, whereas the neural network connecting neighboring ommatidia (~200) generates the wide inhibitory surround. A circadian clock in the animal’s brain transmits signals to the lateral eye at night, changing its structure and function to increase the retinal sensitivity (3) so that the animal can detect mates nearly as well at night as it can during the day (4). Several mechanisms underlying the remarkable nighttime sensitivity have been identified: they are increased photoreceptor gain, decreased photoreceptor noise, decreased lateral inhibition, and increased photon catch as a consequence of an increased acceptance angle for each ommatidium (3). High retinal sensitivity at night is associated with highly variable (“noisy”) optic nerve responses, which result from random photon events at low nighttime levels of illumination. Such noisy neural responses hinder our efforts to measure properties of the nighttime state of the eye and, thus, our development of an accurate cell-based model of retinal function. Our goal is to understand the neural code the eye sends to the brain at night as we have already done for the daytime state of the eye (5, 6). Here we report an analysis of retinal receptive fields and demonstrate how their properties change from day to night.

Our method takes advantage of the remarkable linearity of the responses of the lateral eye to small modulations of the visual input. We employ time-varying sinusoidal stimuli and linear system analysis. Modulated square patterns are presented on a TV monitor having a grey background, with nighttime stimuli attenuated by a 4.25 log unit neutral density filter. After isolating the response of a single optic nerve fiber, we align the animal so that the optic axis of the recorded ommatidium views the center of the pattern. We use animals that had been entrained to the natural
lighting cycle, taking daytime measurements before 1730 h and nighttime measurements from 2200 to 0100. The square pattern is modulated ±10% relative to the background at 1 Hz. Using a strategy previously developed for studying retinal receptive fields in the frog (2), we measured the width of the excitatory center by illuminating the eye with square stimuli of increasing size. Because the inhibitory surround extends into the excitatory center, responses are a mixture of excitatory and inhibitory inputs; but the responses to the smallest square stimuli are predominantly excitatory.

Figure 1A plots the amplitude of modulated optic nerve responses as a function of the visual angle of the square stimuli during the day (gray points) and night (black points). For each data set, the ordinate scale ranges from no response (0%), to the theoretical maximum excitatory response (100%) that would be achieved in the absence of inhibition. The points plotted in Figure 1A show an increase in response for increasing stimulus size, up to 24° of visual angle, beyond which responses decrease because more of the inhibitory surround is illuminated by the square stimulus. The growth of the responses to expanding stimuli (smooth curves) are estimated from the responses to the smallest square stimuli, because the small stimuli minimally activate the inhibitory surround. Assuming that the excitatory center can be well represented by a two-dimensional gaussian function (3), the “pure” excitatory response is proportional to the volume of the excitatory center surface covered by a stimulus. Using this relationship, we estimated the size of the excitatory center based on the recorded responses to the four smallest square stimuli and extrapolated the theoretical maximum response of the excitatory center (100% on the ordinate in Figure 1A; $E_p$ in Equation [1]). The smooth curves plot the theoretical responses, yielding excitatory centers with half-maximal width of 12° during the day (gray curve) and 16° at night (black curve). Because the size of the excitatory center increased at night, the recorded response reached only 85% of its maximum theoretical value before decreasing as a result of surround inhibition. Although the inhibitory surround overlaps the excitatory center, the effects of surround inhibition are minimal for small squares. We therefore attribute the observed changes in Figure 1A to an expanded excitatory field width at night, arising from circadian changes in ommatidial structure, that is, a shift of photoreceptor position and migration of pigmented cells (3).

The vectors in Figure 1B plot the modulated optic nerve response in terms of its phase and amplitude relation to the stimulus—a sine wave with a direction of 0° (vertical) and a length of 1.0. To measure the strength of the inhibitory surround, we first determine the maximal excitatory response, as described above, and plot it as a vector in Figure 1B (open circles). We next determine the response vector for full-field stimulation by modulating the entire TV monitor with a ±10% contrast at 1 Hz (open squares); this vector represents the summed response of excitatory and inhibitory inputs. Finally, we measure the response vector for inhibition by modulating the surround while holding constant the stimulus to the center (crosses). Because the *Limulus* eye responds linearly to small amplitude stimulation, the effects of excitation and inhibition superimpose; i.e., the sum of center and surround response vectors should equal the full-field response. The vector sum of excitatory and inhibitory responses during the day is plotted as a thin line that lies adjacent to the vector for the full-field response (open square) and thus confirms response linearity. We attribute the discrepancy between the vectors at night to response noise at low nighttime light levels caused by random photon events.

**Figure 1.** A: Plot of the response of a single optic nerve (ordinate) as a function of the size of the centrally located visual stimulus (abscissa). Daytime (gray points) and nighttime (black points) responses are normalized to their respective theoretical maximum excitatory response. As expected, the response increases with the size of the stimulus. Solid lines show the growth of the response calculated on the basis of a gaussian-shaped excitatory center. B: Vector plots of responses to illumination of the center (unfilled circle), surround (crosses), and full-field (squares) stimulation. Vectors are plotted relative to the sine wave stimulus, which has an angle of 0° and a length of 1. As explained in the text, responses to center and surround alone predict with reasonable accuracy the response to full-field stimulation, which drives both the excitatory center and the inhibitory surround. Such vector addition confirms the linear properties of the *Limulus* lateral eye. The nighttime vectors show a reduction in the difference between excitation and full-field responses, indicating a decrease in the strength of the inhibitory surround.
From the Hartline-Ratliff formulations of lateral inhibitory interactions in the retina (7), the firing rate of the pth ommatidium \( R_p \) equals its pure excitatory response \( E_p \) minus the sum of inhibitory influences from its neighbors; the inhibition delivered from the jth ommatidium equals the strength of its inhibitory coupling to the pth ommatidium \( K_{pj} \) multiplied by the response of the jth ommatidium. These relationships can be expressed as:

\[
R_p = E_p - \sum K_{pj} \times R_j.
\]

For the case of full-field illumination (8), all ommatidia in the eye receive the same illumination and thus respond at the same rate: \( R_p = R_j = R \). Equation (1) thus simplifies to:

\[
R = E_p - K_{\text{total}} \times R,
\]

where \( K_{\text{total}} \) is the summed total strength of inhibition in the surround, and \( E_p \) is the theoretical, maximum response of pure excitation. All responses are measured to stimuli modulated at 1 Hz. Rearranging Equation (2) gives:

\[
K_{\text{total}} = \frac{(E_p - R)}{R}.
\]

where \( R \) is the magnitude of the vector for full-field illumination (square), and \( E_p \) is the magnitude of the vector for center modulation (circle). From the data in Figure 1, \( K_{\text{total}} \) equals 2.2 during the day, and \( K_{\text{total}} \) is 0.8 at night.

In Figure 1B, the nighttime vector set shows a counterclockwise shift and a reduction in the modulated response amplitudes. The reduction in nighttime vector lengths does not represent a diminution of sensitivity to light, but rather a decreased modulated response to sinusoidal stimulation at 1 Hz. The reduced amplitude and phase shift of the nighttime response vectors result from circadian changes in adaptation and the temporal response properties of the retina.

We conclude, first, that the excitatory center of the receptive field in the lateral eye increases at night. Second, we conclude that the total strength of inhibition in the eye decreases by more than 50% at night. Whether the nighttime decrease in the strength of inhibition also includes a decrease in the size of the inhibitory surround is not yet known, but these results point to a circadian modulation of the synaptic mechanisms that mediate lateral inhibition.

Supported by the National Institute of Mental Health, National Science Foundation, National Eye Institute, Research to Prevent Blindness, and the Central Lions of New York.

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**Frequency Response of Auditory Brainstem Units in Toadfish (Opsanus tau)**

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Toadfish are vocal teleosts. Both males and females produce a pulsed grunt in agonistic contexts. In addition, male toadfish call during courtship, using a “boatwhistle” sound to attract females to the nest. This sound consists of rapidly repeated pulses having a fundamental frequency (F0) of about 130 to 220 Hz in the Woods Hole area, and several higher harmonics up to about 800 Hz (1; Edds-Walton, Mangiamele, and Rome, unpub. obs.). These and other sounds are thought to be detected by the sacculi, which respond to the particle motion component of underwater sound (2). Previous studies (2, 3) showed that primary saccular afferents respond in a phase-locked and directional manner to particle linear oscillations at linear oscillations at 30° azimuth (to the left front and right rear) in the horizontal plane, and at 0° elevation [the best direction for a majority of saccular afferents (2, 4)]. Tone bursts (500 ms in duration, including 20-ms rise-fall times) were presented at equal displacement levels, eight times each, at 50, 65, 84, 100, 141, 185, 244, and 303 Hz. Average spike rate was plotted as a function of tone frequency to define the frequency response areas. Frequency response areas were typically determined at four or more stimulus...
levels within a unit’s dynamic range (generally between \(-10\) and \(30\) dB re: 1 nanometer, root mean square). Ninety-two units of the DON (23 animals, 6 of which were females), and 24 units of the TS (12 animals, 3 of which were females) have been successfully characterized. A unit’s best frequency, or BF, was defined as the stimulus frequency producing the greatest spike rate at the approximate center of the unit’s dynamic range. Frequency response range was defined as the frequency range within which the unit responded at or above 50% of the maximum spike rate (at BF).

Figure 1 shows frequency distributions of BF for the DON units (A-top) and TS units (B-bottom). The lowest BF was 50 Hz (or less), and the highest was 303 Hz (or more). Most BFs were between 100 and 185 Hz. The mode for BF was 185 Hz for DON cells, and 141 Hz for TS cells. There were no differences in the range of BFs for males versus females. The single animal with a BF of 303 was male. While the distributions for both recording sites overlap substantially, relatively fewer units with BFs above 141 Hz were found in the TS. This could be due to sampling error, or it might have resulted from a nonhomogeneous spatial distribution of BF within the TS, which was not explored as systematically as the DON. Frequency response area widths averaged 64 Hz for TS and 117 Hz for DON. This difference probably reflects the lower mean BF for the TS distribution.

Because the fundamental frequency of the boatwhistles recorded in the Woods Hole area varies between 130 and 220 Hz, these results indicate that most, but not all, auditory brainstem cells of the toadfish represent \(F_0\). Thus, contrary to a previous report (8), there is a good “match” between the fundamental frequency of the vocalization and the response of the auditory portions of the brain. In addition, because the highest BF is about 303 Hz, the higher harmonics of the boatwhistle (first harmonic at 360 to 440 Hz), so evident in field recordings (at 19°–22°C; Edds-Walton, Mangiamele, and Rome, unpub. obs.), are probably not represented by the brain, and thus may play no role in boatwhistle perception or other behaviors. The neural representations of the boatwhistle encode the fundamental frequency only. Although not demonstrated experimentally as yet, this frequency is probably equivalent to the rate at which muscular contractions to the swimbladder occur during generation of the sound.

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**Literature Cited**

Males and females behave differently during reproduction. Although sexually differentiated patterns of behavior in vertebrates are clearly regulated by the action of gonadal steroids, the neural mechanisms underlying the expression of sex-specific behavior are largely unknown.

Male and female African clawed frogs (*Xenopus laevis*) produce sexually distinct vocalizations composed of a series of clicks. The fundamental difference between male and female calls is the rate at which the clicks are repeated (reviewed in 1); male calls cover a wide range of click repetition rates (8 to 80 Hz), whereas female calls contain only slow repetition rates (2 to 20 Hz). This behavioral difference can conveniently be reduced to the sexual difference in contraction rate of laryngeal muscle (2), which, in turn, is determined by the sexually distinct firing patterns of laryngeal motoneurons (3). Thus, there is a direct correspondence between the sexually dimorphic patterns of vocalization and the activity of motoneurons.

How, then, do male and female laryngeal motoneurons produce sex-specific patterned activity? While the overall pattern is probably produced by a pattern generator upstream of the motoneurons, the motoneurons themselves may have intrinsic membrane properties that differ between male and female *Xenopus*. Testing this possibility was the goal of this study.

Whole-cell patch clamp recordings were used to characterize the membrane properties of laryngeal motoneurons in n.IX-X of adult male and female *Xenopus*. A thick brain slice preparation of *Xenopus* hindbrain was developed, and the neurons were visualized by IR/DIC microscopy. To facilitate identification, the motoneurons were retrogradely labeled with fluorescent dye (AlexaFluor 594 biocytin, Molecular Probes, Eugene, OR) before the brain was sliced. Responses to hyperpolarizing and depolarizing current steps (200 ms long) by 6 male and 10 female motoneurons (3 male and 6 female frogs), were recorded in current clamp mode. The resting membrane potential, threshold, spike amplitude, and spike half-width were directly measured from voltage traces. The membrane time constant was determined by fitting single exponential curves to hyperpolarizing voltage responses. Input resistance was calculated from the steady-state membrane potential in response to different hyperpolarizing current pulses. The capacitance of the cell was calculated from input resistance and time constant. The peak firing rate was determined by measuring the interval between the first two action potentials in response to the largest depolarizing current (1.5–2nA) applied to each neuron.

Membrane properties of male and female motoneurons are summarized in Table 1. All the properties measured are statistically similar in the two sexes except for the input resistance and the cell capacitance; input resistance is significantly lower, and the cell capacitance is significantly higher in male motoneurons than in female motoneurons. These differences predict that male motoneurons are larger than female motoneurons. A previous study has shown that the dendrites of male n.I-X-X neurons are longer than those of female n.I-X-X neurons, although the somal size is similar in the two sexes (4). The difference in the dendritic arborization may account for the differences in input resistance and cell capacitance in the two sexes. Functionally, sexual differences in the input resistance imply that the male and female motoneurons exhibit different responsiveness to synaptic input.

At depolarized membrane potentials, all the motoneurons showed repeated action potential firing that accommodated over a time course of 100 ms. The peak firing rates, determined by the first two spikes in response to depolarizing currents, were well over 100 Hz in both sexes. To determine whether the neurons could maintain this rapid firing frequency for more than two spikes, four female and two male motoneurons were stimulated with trains of depolarizing pulses at various rates (0.5 ms, 7–10 V, 10 to 300 Hz). Both male and female motoneurons could follow the depolarizing pulses at frequencies of at least 90 Hz. Although the maximum click rate of female vocalizations is 20 Hz, and that of male calls is 80 Hz, the motoneurons of both sexes can fire at a much higher frequency than is required for call production.

Taken together, the results suggest that the laryngeal motoneurons of *Xenopus* do not limit the click repetition rate, and that the motoneurons may be sexually differentiated in their responsiveness to synaptic input.

**Table 1**

<table>
<thead>
<tr>
<th>Membrane properties</th>
<th>Male</th>
<th>Female</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n = 6 )</td>
<td>( n = 10 )</td>
<td></td>
</tr>
<tr>
<td>Resting membrane potential (mV)</td>
<td>-49.1 ± 1.2</td>
<td>-48.1 ± 2.5</td>
<td>n.s.</td>
</tr>
<tr>
<td>Time constant (ms)</td>
<td>9.15 ± 0.91</td>
<td>11.46 ± 1.44</td>
<td>n.s.</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>69.4 ± 3.9</td>
<td>182.4 ± 25.7</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>Cell capacitance (nF)</td>
<td>0.148 ± 0.017</td>
<td>0.066 ± 0.009</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>Spike amplitude (mV)</td>
<td>35.56 ± 3.81</td>
<td>35.94 ± 4.30</td>
<td>n.s.</td>
</tr>
<tr>
<td>Spike half-width (ms)</td>
<td>0.69 ± 0.10</td>
<td>0.67 ± 0.11</td>
<td>n.s.</td>
</tr>
<tr>
<td>Threshold (mV)</td>
<td>-29.11 ± 10.25</td>
<td>-36.14 ± 3.12</td>
<td>n.s.</td>
</tr>
<tr>
<td>Highest firing frequency (Hz)</td>
<td>192.17 ± 29.47</td>
<td>257.40 ± 25.86</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

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What information does the eye send to the brain when an animal sees? We are exploring this question with the relatively simple visual system of the horseshoe crab, Limulus polyphemus. By combining cell-based computational models of the retina with single-cell electrophysiology, we have examined the optic nerve code underlying Limulus vision during the day in the animal’s natural habitat (1).

Field studies during the animals’ mating season show that male horseshoe crabs use vision to find mates and do so about equally well day and night (2). We attribute their remarkable nighttime vision to a circadian modulation of the sensitivity of their lateral eyes (3). At night, efferent optic nerve fibers carry signals from the circadian clock in the animal’s brain to its eyes, increasing nighttime retinal sensitivity as much as 1,000,000 times. The increased sensitivity nearly compensates for the average 1,000,000-fold decrease in ambient light intensity after sundown. Here we investigate optic nerve activity recorded from the animal in its habitat, both day and night, with emphasis on signals that convey information about potential mates at night.

A convenient method for recording what the horseshoe crab sees underwater is to mount a miniature video camera, “CrabCam,” on the animal (1). This documents the crab’s eye view during the day, but not at night when light levels fall below the camera’s sensitivity. To investigate the optic nerve responses of an animal in its natural habitat at night, we used a repetitive, artificial stimulus that simulates the movement of a potential mate within the animal’s visual field. The stimulus is a rotating grey cylinder (30 cm in diameter, 15 cm in height) with a black sector (30 cm in width) that simulates the size of a typical female horseshoe crab. The cylinder was placed 1 m from the crab and rotated by hand (4 – 8 rpm), which simulates the movement of a potential mate within the animal’s habitat.

The video frames were taken 6 s after the beginning of the response records (arrows), when the grey-black edge of the cylinder began to enter the field of view of the recorded ommatidium from the right. In both cases, the black sector evoked clear decreases in response, with the larger decrease recorded when the cylinder was closer to the animal and water turbidity was minimal. The top “Day” response was recorded at 1630 h and the second was recorded at 1800 h. In both cases, the setup was bathed in direct sunlight. After the second record was recorded, the animal and cylinder were left underwater as nightfall approached.

The experiment was then repeated several hours later after sundown (2030–2100 h) but without CrabCam recordings because of insufficient lighting. Figure 1 (“Night”) displays the responses of the same single optic nerve fiber to nine sequential rotations of the cylinder (thin black traces). The heavy black trace gives the average of the nine responses. Note that the individual responses

are highly variable relative to those recorded during the day, and that the average response rate to the grey sector is about 3 impulses/s which is 6-fold lower than the mean daytime response rate of about 18 impulses/s (middle trace). We attribute the highly variable response rates to random photon events occurring at the very low nighttime levels of illumination. The nighttime sky during this experiment was heavily overcast and lacked moonlight. From radiometric measurements we estimate that ambient light decreased by about $10^6$ to $10^7$ relative to daytime levels. The circadian increase in lateral eye sensitivity cited above nearly compensates for such large reductions in ambient lighting. Experiments in the laboratory (R. Barlow and F. Dodge, unpub. obs.) indicate that the average response to the grey sector of $\sim 3$ impulses/s is about 50% lower than expected for the low nighttime levels of illumination. The surgery performed to isolate the single optic nerve fiber may have partially damaged the fragile efferent fibers that carry the circadian clock’s signal from the brain to the eye; as a consequence, the lateral eye may not have received the normal efferent input and thus the retina may not have shifted completely to its fully sensitive nighttime state. Nevertheless the eye’s circadian increase in sensitivity was sufficient to detect the rotating black sector of the cylinder, which mimics a moving mate.

Computational analyses of visual processing in the *Limulus* brain indicate that retinal inputs may sum at the first synaptic level

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**Figure 1.** The responses of a single ommatidium to an underwater rotating visual stimulus. The records on the left plot the instantaneous frequency (reciprocal of the interval between adjacent optic nerve impulses) as a function of the rotation of the stimulus shown on the right. The speed of rotation is approximately the same for all records. The visual stimulus is aligned so that the optic axis of the recorded ommatidium is centered on the rotating cylinder with its field of view lying within the black sector. At a distance of 1 m the black sector intercepts the optic axis of about 9 ommatidia. The “Day” responses were recorded between 1630 and 1800 h, and the “Night” responses were recorded between 2030 and 2100 h. The top video frame was taken in the clear water of Great Harbor, Woods Hole, Massachusetts, where the grey/black sectors of the cylinder had a contrast of 69% [contrast $= (L_{\text{Grey}} - L_{\text{Black}})/(L_{\text{Grey}} + L_{\text{Black}})$]. The second video frame was taken near Stoney Beach, Woods Hole, where turbid water reduced the contrast of the grey/black sectors to 26%. Arrows indicate the times at which the underwater scenes to the right were videotaped. At these times the black sector begins to enter the field of view of the recorded ommatidium, reducing its response rate. The “Day” records are responses to a single rotation of the cylinder in a right to left direction (loop with arrow). The “Night” records show responses to nine consecutive rotations of the cylinder (thin black traces; period of rotation $\sim 16$ s) and their average (thick black trace). The peaks and valleys of the thin black traces reflect the highly variable rate of discharge of the single optic nerve fiber under low nighttime levels of illumination.
(5). Spatial summation across a matrix of 5–10 ommatidia significantly increases the signal-to-noise properties of responses recorded at night. Indeed summing seven sequential optic nerve responses to the rotating cylinder yielded a relatively noise-free response.

These experiments represent our first attempts to analyze lateral-eye responses of Limulus at night in the animal’s natural habitat. The use of a periodic stimulus obviated the need for video documentation of the visual stimulus, which is not feasible under nighttime lighting conditions. With this technique, we successfully recorded visual responses in the animal’s habitat and found that the lateral eye transmits information to the brain about mate-like objects at night under dark overcast skies. Under such conditions Limulus could see what we could not.

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Do the Properties of Underwater Lighting Influence the Visually Guided Behavior of Limulus?
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In the spring, horseshoe crabs, Limulus polyphemus, migrate to the water’s edge along the East coast of the United States to pair off and build nests (1). As they enter a nesting area, males use their lateral eyes to locate mates both day and night (2). They approach females and objects resembling them, such as rocks, patches of seaweed, or mate-like objects. What does a male see in a female? Her size and contrast are two important factors. Males are attracted to objects that approximate the size of females. They orient toward mate-like objects at distances up to 1.2 meters, detecting higher contrast objects better than lower contrast ones (3). How do the properties of underwater lighting in the animal’s natural habitat influence whether a crab finds a mate during the day or at night? The approximate 1,000,000-fold reduction in ambient lighting after sundown has no appreciable effect. Their remarkable visual performance results in part from a circadian increase in lateral eye sensitivity of as much as 1,000,000 times at night (4). In this paper we consider another property of the animal’s underwater habitat, termed “strobic lighting.”

In the shallow waters of nesting areas, overhead waves act like lenses, creating moving beams of sun- and moonlight that reflect off the sandy bottom and submerged objects. On average, the peak intensity of these beams is about three times that of ambient illumination. The fields of view of single ommatidia are wider than the moving beams of light. Because ommatidia sum the illumination within their field of view, the amplitude of modulation of the light beams reaching the underlying photoreceptor cells decreases to about 70% contrast. The strobic illumination by the beams strongly modulates the firing rate of an ommatidium, with peak firing rates reaching three times the mean (5). Such strobic illumination might be expected to enhance the detectability of underwater objects, such as potential mates. Indeed, an earlier study suggested that strobic conditions enhance the visibility of low contrast mate-like objects, and that without strobing, Limulus is attracted to higher contrast objects (6). We have further explored the influence of strobic lighting by carrying out more field studies and combining the results with those collected over the past five years.

We investigated the visual performance of Limulus during their springtime mating seasons at Mashnee Dike, Bourne, and North Monomoy Island, Chatham, both located in Massachusetts. Our study and those of previous years were carried out day and night under various weather conditions ranging from dense cloud cover to clear skies, yielding 106 to 102-fold diurnal changes in the intensity of ambient illumination, with an average change of about 104. In all our studies, a modified two-alternative forced choice technique adapted from human psychophysics was used (7). As shown in Figure 1, we placed on the sandy bottom a clear Plexiglas chute with a funnel at one end and a narrow chute at the other. Crabs entered the funnel and, upon exiting the chute, were presented with the choice of a black or gray female-sized object; these were located 1 m from the exit of the chute and 1 m from each other, creating an equilateral triangle. The objects were either a hemisphere (diameter of 0.3 m) or a cylinder (height of 0.15 m, diameter of 0.3 m), both approximating the size of an adult female horseshoe crab. The objects were switched periodically during an observation period to avoid any effects of directional bias in behavior. Their black and gray tones represent the greatest range of contrast of the female carapace (8). The black object has a negative contrast of 37% against the background of sand and seawater, and the gray object has a positive contrast of 35%. Animals exiting the chute either approached and contacted one of the two targets or swam by them. In 1999 and 2000, about 60% of animals exiting the chute did not approach or contact either submerged object. The animals passing by both objects were not recorded in the years

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2 Allegheny College, Meadville, Pennsylvania.

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preceding 1999. More than 99% of the animals studied were males, because animals in amplexus were prevented from entering the funnel, and <1% of single animals in nesting areas are females (1). We could not determine whether a specific male crab passed through the chute more than once in a single observation period, but since animals in nesting areas are abundant, such events are unlikely to have occurred. When an animal exited the chute, observers noted whether the lighting conditions were strobic or nonstrobic. In 1995 through 1998, nonstrobic conditions occurred naturally day and night, under cloudy skies or in calm water. In 1999 and 2000, we controlled strobing by placing a clear-bottomed box on the surface of the water above both targets. The Plexiglas bottom of the box prevented rippling wave action, thereby eliminating strobic lighting of the underwater scene (See Fig. 1).

Table 1 summarizes the data collected in 2000 and during the five previous years. Taken together, the data for all six years (“Total” in Table 1) indicate that, under nonstrobic conditions, there is no significant difference between the number of animals attracted to the two objects day or night (P-values of 0.76 and 0.077 respectively, as determined by the χ² test). Under strobic conditions, significantly more animals, 69 or 24% more, approached the gray object during the day (P < 0.00005). The greater number of animals, 12, attracted to the gray target at night under strobic conditions, was not significant (P > 0.4).

These field studies show that when the distribution of illumination in the animal’s natural habitat is uniform (nonstrobic conditions), the animals detect black and gray mate-like objects about equally well day and night. This is understandable because the black and gray objects have about the same absolute contrast, 37% and 35% respectively, against the underwater background. Under strobic conditions, significantly more animals are attracted to the gray object during the day, but not at night. This is also understandable because, as described above, the moving, underwater light beams increase the contrast of the gray object, but not the black one. Indeed optic nerve recordings in the animals’ natural habitat reveal bursts of activity in response to gray objects illuminated by strobic light (5). Why the gray objects are not more attractive under strobic conditions at night is not understood. The highly variable optic nerve discharge resulting from random photon events at low levels of nighttime illumination (9) might be masking the bursts of activity generated by strobic lighting. We conclude that the properties of ambient lighting can affect an animal’s vision in its natural habitat, particularly during the day. Limulus is not unique. Strobic lighting appears to have a prominent role in the visual performance of other marine animals (10, 11).

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**Startle Responses of Fish Without Mauthner Neurons: Escape Behavior of the Lumpfish (*Cyclopterus lumpus*)**

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Fast start escape responses are the primary behaviors used by fishes to avoid an attacking predator. Of particular importance is the C-start type of fast start (reviewed by 1, also see 2, 3). During a C-start the fish rapidly turns away from a threatening stimulus into a “C” shaped body bend, called stage 1. Frequently, stage 1 is followed by a tail stroke to the opposite side of the body, stage 2, which propels the fish away from the stimulus. The C-start is initiated by the Mauthner cells, a pair of large reticulospinal interneurons (4, 5). Each M-cell has a large axon that crosses the body midline and extends the length of the spinal cord, exciting motoneurons that innervate the lateral muscle. In response to a stimulus from the right side of the body, the right M-cell fires an action potential that propagates rapidly down the axon to cause nearly simultaneous contraction of muscle on the opposite side of the body from the M-cell soma and the “C” bend away from the stimulus (6, 7).

Although Mauthner cells have been identified in a large number of taxa broadly representing the phylogenetic diversity of actinopterygian fishes, a few species appear to lack these neurons (8). This study examines the startle behavior of one such species, the lumpfish (*Cyclopterus lumpus*). Two specific questions are addressed. First, do lumpfish have a startle response that is distinct from routine swimming? If so, how does the behavioral pattern and performance compare with the M-cell initiated C-start of other fishes?

The startle response was examined in larval lumpfish rather than in mature individuals. The larval lumpfish have a more generalized morphology than mature lumpfish, and so it was thought that the response of the larvae to a startle stimulus may be more easily compared to other species. Additionally, it seemed that if the lumpfish were to have high performance behavioral responses to predation, it would be seen in the larvae because of greater vulnerability to predators due to less developed morphological defenses. It is possible that M-cells are present in larval lumpfish and are reduced or lost during development; however, morphological examination of the reticulospinal neurons of the larval lumpfish (*n = 30*) with retrograde labeling has not identified Mauthner neurons or homologous cells.

For studies of behavior, lumpfish (*n = 12; 6.2 ± 1.0 mm, total length*) were hatched from eggs collected off the coast of Gloucester, Massachusetts, at approximately 6 m depth. Eggs and larvae were maintained in a 10-gallon aquarium with flow-through seawater chilled to 11°C. Behavioral trials were conducted within a week of hatching. A tactile stimulus—touching the head with a fine gauge wire—was used to elicit startle behavior which was filmed in a small petri dish (3.5 cm diameter). The responses were captured on high-speed video (1000 Hz) taken with an EG&G Reticon digital camera imaging through a Zeiss Stemi SR microscope. Three trials from each fish (36 total trials) were analyzed with Microsoft Excel 98 and Scion Image 1.6. Parameters examined were the angles of head movement during stage 1 and stage 2, the latency between stimulus and response, and the durations of stages 1 and 2.

The larval lumpfish respond to the stimulus with a C-start behavior pattern (Fig. 1A). Fish turned tightly away from the stimulus direction in stage 1 [Fig. 1A, left column (0–24 ms)] with an average stage 1 angle of 146° ± 23°C degrees. Stage 1 was consistently followed by a stage 2 tail stroke [Fig. 1A, right column (24–56 ms)] and movement away from the stimulus. The stage 2 angle, generally in the opposite direction of the stage 1
was consistently smaller than that of stage 1 (stage 2 angle = 50° ± 30°). The movement angles made by larval lumpfish during the C-start are comparable to those of other species (Fig. 1B; e.g. 9, 10, 12, 13).

Several important fast start performance variables are the latency of response to the stimulus and the duration of the kinematic stages. The latency between stimulus and initiation of movement of an M-cell initiated startle can take less than 4 ms (9) and the duration of the response is generally less than 100 ms (1). The latency of the lumpfish, recorded for a subset of the trials (one from each of 10 individuals) was 9.6 ± 2.1 ms. It was considerably longer than that of the larval zebrafish (3.9 ± 0.2 ms) (9). The duration of stage 1 of the larval lumpfish was 22.8 ± 5.2 ms, and the duration of stage 2 was 26.3 ± 6.8 ms. Because the duration of the fast-start stages changes with size (11) and developmental stage (10), direct comparisons among species are difficult. Still, the durations of kinematic stages 1 and 2 of the larval lumpfish are in the same range of values as other immature fishes; all under 5 cm (Fig. 1B; 9, 10, 12). The total duration of the fast start (stages 1 and 2) for the larval lumpfish is shorter than the fast start duration of most larger fishes (reviewed in 1).

Although the lumpfish has a longer response latency to a startle stimulus than zebrafish larvae, the C-start of the larval lumpfish—in pattern and in the duration of response—has the characteristics of the M-cell initiated C-start. One explanation for the similarities in the startle response among taxa is that the Mauthner cell and its homologs are present in the larval lumpfish but have not yet been identified. Another is that alternative neural circuits can generate rapid C-start behavior and that the Mauthner cell and its homologs are most critical for rapid initiation of movement. If so, such mechanisms may be taxon specific since ablating the Mauthner cell and its homologs in the larval zebrafish results in a significant decrease in performance (9). The presence of a rapid C-start type escape behavior in the lumpfish, a species that appears to generate the fast start behavior without the Mauthner cell system, provides an exciting opportunity for comparative examination of an evolutionarily conserved neural and behavioral system.

I thank S. Van Sant and J. Fetcho for their contributions of fish and equipment, respectively. Thanks to S. Zottoli and M. Westneat.

Figure 1. A. A typical startle response of larval lumpfish (Cyclopterus lumpus). Stage 1, the tight “C” bend away from the stimulus, lasts 24 ms (column 1) and stage 2, the first propulsive tail stroke, follows from 24 to 56 ms (column 2). Data for the angle of movement and kinematic stage durations are shown in B, with comparative data from brown trout (Salmo trutta), chinook salmon (Oncorhynchus tshawytscha), coho salmon (Oncorhynchus kisutch) (10, minimum values in scaling relationships), zebrafish (Danio rerio) (9), and herring (Clupea harengus) (12).
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Memory Consolidation in *Hermisenda crassicornis*

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Experiments with shell-less molluscs (*Aplysia* and *Hermisenda*) have revealed a number of processes that underlie learning by these organisms and also by some vertebrates. *Hermisenda*, for example, shows significant Pavlovian conditioning capabilities (1). Follow-up investigations on these molluscs dealt with the sensory stimuli needed for short-term memory (STM) and long-term memory (LTM) (2). The relationship of the two memories with *in vitro* changes in excitatory post-synaptic potentials (EPSPs) have also been investigated in the neural networks of these organisms (3). Many studies of the molecular aspects of these two different memory regimes have led to quite detailed descriptions of the events (4, 5, 6).

Both *Aplysia* and *Hermisenda* have been tested for their recall of induced behavioral modifications after one, two, or many conditioning events (CEs). In *Aplysia*, the EPSP component of learning produced by 1 CE was compared to that produced by 5 CEs (3). In *Hermisenda*, the comparison was made between 2 CEs and 9 CEs (2). Five to ten minutes after finishing one or two conditioning events, both animals exhibited significant behavioral recall (*i.e.* STM); but there was no recall after an hour or more (*i.e.* no LTM). The larger numbers of CEs, however, did induce LTM in both species.

Since STM and LTM are clearly responding to a different set of conditions, we focused first on what might inhibit or block STM. This problem was partially anticipated in 1900, according to McGaugh (7) who cited Muller and Pilzecker as having found that “memory of newly learned information was disrupted by the learning of other information shortly after the original learning” (8).

This concept led us to test, in *Hermisenda*, whether STM recall (at 5 min) might be blocked simply by the input of additional information (*i.e.* extraneous sensory stimuli) if the latter were applied within the first 5 min after conditioning. The initial results of the blocking experiments, which showed that the simple sensory inputs blocking STM also blocked LTM, then led to the hypothesis that temporal consolidation of LTM could be detected by measuring when the blocking sensory input was no longer effective.

*Hermisenda* (Sea Life Supply, Sand City, CA) were tested with 2 and 9 paired CEs for induction of STM and LTM. Conditioning events consisted of exposing the animals to 6 s of bright, white light (CS) explicitly paired with 4 s of strong orbital agitation (US) following a 2-s onset delay with an inter-trial interval of 1 min. Recall of the behavioral modification induced by associative conditioning was assessed by recording the animal’s change in foot length when presented with 6 s of light alone. The conditioned response (CR) was foot contraction, the unconditioned response (UR) was foot elongation (9). Two paired conditioning events initiated behavioral recall after 5 min but not after 90 min; the LTM input of 9 pairings was recalled at both 5 and 90 min (Fig. 1A). The small and non-overlapping S.E.Ms for each point indicate statistical significance (*P* = 0.01, *t* = 3.18).

After giving the animals the paired CS and US stimuli leading to STM, we tested two simple paradigms of blocking sensory stimuli. The first was a modification of the conditioning stimuli: dim orange light and very slow orbital rotation. The second blocking stimulus tested consisted of rotating the tray containing the animals upside down and, after 5 s, rotating it upright again (rotational block). Both experimental paradigms blocked STM and LTM (Fig. 1B).

To determine the temporal specificity of LTM in *Hermisenda*, the following experiments were done. Animals were trained with 9 CEs, and the CR was measured at the usual 90 min. However, at selected time intervals (2, 25, 50, 55, 60, 65 min) post-conditioning, the animals were rotationally blocked. Control animals received only the 9 paired CEs. When the animals’ behavior was plotted, a clear and decisive LTM consolidation interval in *Hermisenda* appeared; consolidation occurred between 55 and 60 min (Fig. 1C). Presentations of rotational blocking prior to 55 min totally blocked memory consolidation. However, the stimulus given after 60 had no blocking effects, and the animals demonstrated the CR. The consistency of and surprisingly little variability in the response among the majority of the animals indicated the robustness of the paradigm. When the data were analyzed with *t*-test and *F*-test statistics, they were found to be highly significant, whether compared between data points or to zero (*P* = 0.001, *t* = 15.24; *F*-value, inf).
As a secondary result of these experiments, we could assess the degree to which an animal’s training is modified by extraneous handling. The less than optimal (non-significant) training results of animals given 9 CEs and tested after 90 min (i.e. 9CE90 animals; Fig. 1B) could be directly attributable to the effects of handling. In future behavioral experimental protocols, as well as in routine animal training procedures, the potential for introducing extraneous blocking stimuli that could confound results must be eliminated.

Memory consolidation has long been recognized to depend on the species being studied, the nature and the frequency of the instructional inputs, and the kind of blocking activity used to determine when the consolidation is completed (7). Our results thus far apply only to the simplest of conditioning stimuli. However, if the concept of consolidation time applies to humans, then awareness of it could help with schooling in two ways: by alerting teachers to the possible blocking of learning by presenting unrelated materials too close to the main point, or by waking teachers to the need for waiting past the consolidation time before asking complex questions about what had just been presented to the students. Our best estimate, derived from the literature, is that human consolidation time for simple inputs is about 6 to 10 min (10).

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response (CR). The numbers beside the points (e.g. 25.70) signify the number of animals measured, and observational differences averaged. (B) The effects of the two experimental blocking stimuli applied during the first 5 min after training and tested for inhibition of short-term memory (STM) and long-term memory (LTM). Designations are as in Figure 1A with the addition of B1, first blocking sensory stimuli (dim light plus slow agitation); B2, second blocking stimulus consisting of vertically rotating the animals upside down for 5 s, then re-righting them. Both blocking paradigms interfered with STM and LTM. Weakened conditioning response of 9CE90 was due to the blockade of memory induced by handling. (C) Temporal specificity of memory consolidation in Hermissenda. Percent foot changes measured 90 min after training with 9 CEs, but with the second blocking stimulus (B2, vertical rotation) applied at designated times after the end of training. Memory consolidation appeared to occur between 55 and 60 min. Statistical analyses using t-tests and F-tests were highly significant (P = <0.001).
Mechanisms of Spontaneous Miniature Activity at CA3-CA1 Synapses: Evidence for a Divergence From a Random Poisson Process

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Most of the CNS synapses investigated so far release quanta in a spontaneous manner. Since the pioneering work of Fatt and Katz (1), spontaneous release of synaptic quanta is considered a probabilistic process where each quantum is randomly discharged at individual release sites. Release sites are assumed to behave independently and discharge quanta at a very low and stable rate. This hypothesis has been confirmed by a large number of observations at the neuromuscular junction and at other peripheral terminals (1–2). Regrettably, it is unclear that such a description of the release process applies to small CNS synapses. To investigate this problem, we used whole-cell recordings to study the stochastic properties of spontaneous release at hippocampal synapses. In these experiments, clustering of miniature events (minis) was a consistent observation. In about 60% of the experiments, multiple decaying exponentials were required for best fit of interval distributions, and this facilitatory behavior—which could not be accounted for by random coincidences—was independent of calcium elevations in the cytosol. For these experiments, postnatal CA3-CA1 hippocampal cultures were prepared from P4-5 neonatal rats essentially as previously described (3). Neurons were used for synaptic experiments 10–21 days after plating. Whole-cell recordings of miniature currents were also made, as previously described (3).

Hippocampal neurons were continuously perfused with a bath solution containing (in mM): NaCl 119, KCl 5, CaCl2 2, MgCl2 2, HEPES 25, glucose 30, picrotoxin 100 μM (Sigma), APV (D-2-amino-phosphonovalerate) 25-100 μM, tetrodotoxin 0.5 μM, adjusted to 305 mOsm and pH 7.4. Patch electrodes (2–5 MΩ) contained (in mM): Cs-gluconate 110, MgCl2 5, NaCl 10, EGTA or BAPTA 0.6–10, ATP 2, GTP 0.2, HEPES 49, adjusted to pH 7.2 and 290 mOsm. Current traces were digitized off-line from the magnetic tape at 10–70 KHz after low-pass filtering at 3–5 KHz. Miniature events were detected semiautomatically (3). Intervals between events were log-binned and plotted on a log-log scale: the bin content was normalized for the bin width (4). The resulting histograms were fitted with nested models made by the sum of exponentials with a Simplex algorithm and a maximum likelihood estimator. The minimum number of exponential components was chosen using the log-likelihood-ratio test. Averaged values are reported as the mean ± s.e.m., and statistical comparisons were obtained using an independent Student’s t test, unless otherwise indicated. In these conditions, when the occurrence of spontaneous events was analyzed, clusters of minis (i.e., very brief episodes with a few consecutive quantal releases) were found to be a very consistent observation. The statistical significance of this finding can be tested by analyzing the distributions of inter-event intervals from large data sets. According to predictions from Poisson’s law, a frequency distribution of random intervals should display a mono exponential profile:

\[ F(x) \approx \exp(-\lambda x) \]

where \( F(x) \) is the probability of having an interval greater than \( x \), and \( \lambda \) is the mean mini frequency. Log-binned frequency distributions of mini-intervals indicated (4) that in most cases (\( n = 14/24; P < 0.05 \), log-likelihood ratio test), multiple decaying exponentials are required for optimal fit of interval distributions. This indicates a clear divergence of spontaneous exocytosis from a random Poisson process (Fig. 1, A-B). The best fit of the interval distributions indicated that the area underneath the fast or bursting component (afast) could be as high as 66% or as low as 3% (mean afast = 12 ± 4%; \( n = 14 \)) (Fig. 1, A-B). Since miniature events arise from a large population of independent synapses, could this divergence arise simply from the temporal averaging? The answer is “No,” according to the following argument. If each synapse generates spontaneous events according to a Poisson process, then the probability of finding \( k \) events in the time interval \( \Delta t \) is:

\[ P(k) = \frac{\exp(-\mu \cdot \Delta t) \cdot \mu^k}{k!} \]

where \( \mu \) is the mean Poisson rate at the ith synapse. With a population of \( N \) independent synapses, the occurrence of minis at the soma will also be a Poisson process with a single parameter \( \mu \) which is just the sum of the individual parameters:

\[ \mu = \sum_{i=1}^{N} \mu_i \]

Therefore, based on these simple mathematical considerations, if every synapse made onto an individual neuron is releasing in a random manner, whole-cell mini interval distributions should display a single exponential component. Moreover, this conclusion would also be valid in the presence of a large variability in spontaneous quantal rates at different synapses, as previously reported in the same system (5). In agreement with these expectations we have used a technique that permits to us record minis from individual hippocampal synapses and have found that, even at the level of a single terminal, the generation of quanta diverges from a random memory-less Poisson process (Abenavoli et al., unpub.).

A transient up-modulation of quantal discharges, such as the

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one observed in the fast component of mini interval distributions, might result from some sort of transient change in presynaptic Ca$^{2+}$ levels (see ref. 6 for review). We have therefore tested the effects of cadmium (50 μM), a broad spectrum Ca$^{2+}$ channel blocker. When cadmium was applied, no effects on mini frequency and mini amplitude were detected. In 9 cells, the average mini frequency in control conditions was 2.51 ± 0.75 Hz, and it was 2.56 ± 0.74 Hz after the application of cadmium. In these experiments, when log-binned distributions of mini-intervals were constructed, if multiple decaying exponentials were required for optimal fit in control conditions, they were also required in the presence of Cd$^{2+}$ (n = 4/4; P < 0.01). We also examined the effects of BAPTA, a high affinity, fast-binding Ca$^{2+}$ chelator (7). BAPTA was introduced in all synaptic terminals impinging upon a postsynaptic neuron by perfusing those neurons with the membrane permeable analog BAPTA-AM while recording synaptic events. Long-term application of BAPTA (>20 min) (in the presence of tetrodotoxin) produced no significant effect on mini frequency (⟨f$_{\text{Control}}$⟩ = 2.14 ± 1.30 Hz, ⟨f$_{\text{Bapta}}$⟩ = 1.74 ± 1.56, mean ± sd; n = 7). Importantly, interval-distributions of minis displayed no detectable change after the BAPTA treatment (n = 3/3; P < 0.05; a$_{\text{Control}}$ = 8 ± 3%; a$_{\text{Bapta}}$ = 8 ± 3%). Taken together, these observations rule out a role in the divergence from Poisson’s statistics. The histogram presented was best fitted by the sum (solid line) of two decaying exponentials (dotted lines); B) Summary data for the area (a$_{f}$) of the short-interval component (range a$_{f}$ = 3–66%, mean value = 12 ± 4%; range $\tau_f$ = 1.56 – 48.64 ms, mean value 20.37 ± 4.07 ms).

Figure 1. Distribution of intervals between minis at hippocampal synapses. A) Minis were acquired in voltage clamp using the Whole-cell recording configuration. Short trains of minis could be seen consistently under these conditions. In these experiments multiple exponentials were always required for best fit of mini-interval distributions indicating a divergence from Poisson’s statistics. The histogram presented was best fitted by the sum (solid line) of two decaying exponentials (dotted lines); B) Summary data for the area (a$_{f}$) of the short-interval component (range a$_{f}$ = 3–66%, mean value = 12 ± 4%; range $\tau_f$ = 1.56 – 48.64 ms, mean value 20.37 ± 4.07 ms).

Nonetheless, we can speculate that, since minis keep occurring in the absence of any incoming electrical activity, trophic support through minis would circumvent requirements for Hebbian mechanisms to maintain some forms of synaptic plasticity (8). In particular, the rapid discharge within a burst would certainly lead to a temporal summation in the postsynaptic spine and dramatically increase the probability of calcium influx through postsynaptic NMDA channels. The input-output properties of CNS synapses are an additional consideration.

The results presented might also be relevant to the hypothesis that the release of multiple vesicles is happening under some conditions and in some neuronal systems during spontaneous and evoked exocytosis (9–12). Regardless of the frequency of multivesicular exocytosis, this could certainly have an impact on the synaptic input-output characteristics of hippocampal synapses, since glutamate AMPA receptors are not saturated by the content of a single vesicle (3).

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UV-B Induced Damage to the Skin and Ocular System of Amphibians

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The depletion of stratospheric ozone and the consequential increase in ultraviolet (UV-B) radiation reaching the Earth’s surface may be partly responsible for the recent declines in some amphibian populations (1). Present or predicted UV-B levels for the next decades in northern latitudes can cause pronounced mortalities in amphibians during early development both under laboratory and semi-natural conditions (2–6). However, except for limb and spinal malformations (7, 8), observable damage at the systems level has been seldom described. Here, we report on the incidence of skin burns and eye cataracts in tadpoles exposed to enhanced UV-B levels under semi-natural conditions.

The tadpoles examined in this study originated from experiments carried out in a forest clearing near Victoria (British Columbia, Canada) using containers filled with pond water (4). Two species of frogs (Hyla regilla and Rana aurora) were raised from eggs under one of three light regimes: (1) ambient sunlight, (2) sunlight blocked for UV wavelengths less than 450 nm (control), or (3) sunlight enhanced from 4.8% to 23% UV-B (280–320 nm) over ambient levels at noon, depending on atmospheric conditions (from sunny to overcast days, respectively [5]). The enhanced UV-B treatment was obtained by placing fluorescent UV-B lamps, with peak output in the range of 280–315 nm, over the appropriate containers. These lamps were on from 0900–1700 h each day, resulting in a mean hourly increase of approximately 30% UV-B radiation per day over ambient levels for the duration of the study. Tadpoles were collected from each treatment during weeks 3 and 4 after hatching and examined under a dissecting microscope for integument burns. To assess damage to retinal photoreceptors, the retinas from five animals collected during week 4 were prepared for electron microscopy. This procedure involved enucleation of the eyecup and fixation in primary fixative (2.5% glutaraldehyde, 1% paraformaldehyde in 0.06 M phosphate buffer, pH 7.3); post-incubation of extracted retinas in secondary fixative (1% osmium tetroxide) for 1 h at 4°C; dehydration of the tissue through a series of ethanol solutions of increasing concentration; and embedding in Epon plastic. Thin (75 nm) radial sections were cut, exposing photoreceptors from the centroventral part of the retina along their lengths. These sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with the transmission electron microscope.

The numbers of dead embryos and larvae were monitored throughout the study (4). Mortalities during the study can be summarized as follows. Although significantly higher egg mortality was observed in the enhanced UV-B treatment for R. aurora only, tadpoles of both species suffered high mortality in the enhanced treatment: only 2.6% of R. aurora and 18.4% of H. regilla survived 1 month after hatching, compared to 55% and 51.7% in the ambient treatment, and 43.8% and 65% in the control.

In the control and ambient treatments, the majority of animals had clear lenses (Fig. 1a). Of the controls, only 3 of 10 R. aurora and 2 of 10 H. regilla tadpoles examined during weeks 3 and 4 after hatching showed signs of lens opacities. The extent of these opacities was smaller than that presented in Figure 1b. In the ambient treatment, 3 of 9 R. aurora and 1 of 10 H. regilla showed similar small lens opacities. In the enhanced UV-B treatment, however, 16 of 20 R. aurora and 12 of 16 H. regilla tadpoles showed prominent lens opacities. Thus, for both species, more tadpoles raised under the enhanced UV-B treatment showed lens opacities than did those from the control or ambient treatments (R. aurora $\chi^2 = 11.4, \text{df} = 2, P < 0.005$; H. regilla $\chi^2 = 13.4, \text{df} = 2, P < 0.005$). Large opacities, a sign of advanced cataracts, were particularly visible among R. aurora tadpoles (Fig. 1c). Substantial skin burns were also present in 6 H. regilla and 9 R. aurora tadpoles from the enhanced UV-B treatment (Fig. 1d); however, this skin damage may have been amplified by fungal infections (9). No skin sores were found in tadpoles from the ambient and control treatments.

Electron micrographs revealed single cones that were similar in all animals irrespective of treatment (Fig. 1e). The outer segments of cones (Fig. 1f), which contain the light-sensitive visual pigments, were not appreciably different from those of fish at early developmental stages (personal observation). Our data, therefore, suggests that the damage from enhanced UV-B radiation to the ocular system of amphibians occurs primarily in the lens. It may be that the early onset of lens opacities prevents substantial levels of UV-B radiation from reaching the photoreceptor layer of the retina and disrupting cell structure. However, other explanations, including the involvement of repair mechanisms, may also account for these results.

The incidence of skin sores and cataracts, as observed in our UV-B enhanced treatment, can have important consequences for tadpoles in nature. Animals with cataracts are incapable of forming a clear image in the retina due to scattering of light within the lens, hence their foraging and predator avoidance capabilities are greatly reduced. In addition, the skin sores can be infected by parasites, thereby further increasing tadpole mortality (9).

In many regions of the world, the removal of riparian vegetation and creation of large clearcuts increases the exposure of small water bodies, used by amphibians for oviposition, to UV-B and total radiation levels (10). Tadpoles developing in such habitats might be exposed to UV-B levels sufficient to cause the type of damage reported here. Monitoring the incidence of cataracts in clearcuts and forest pools could potentially be used as an indicator of biological effects of increased UV-B radiation.
Figure 1. Photographs of (a) *Hyla regilla* tadpole with clear lens; (b) *H. regilla* tadpole with lens opacity (arrowhead); (c) *Rana aurora* tadpole showing extensive cataracts (arrowhead); (d) lesion area of *R. aurora* tadpole exposing dorsal musculature; (e) *R. aurora* cone photoreceptor showing normal inner and outer segments (is and os respectively), bar = 1.2 μm; and (f) details of two cone outer segments, bar = 0.6 μm. No damage was readily apparent in the photoreceptor layer of tadpole retinas from any of the treatments.

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The cuticular secretion can be provoked by housing healthy adults in the presence of decaying fish (3). The secretion from the hypodermal glands was scraped from the dorsal cuticle of the Limulus polyphemus. One of the major identified causes of mortality of adult horseshoe crabs is the erosion of the cuticle that follows its colonization by green or blue-green algae (1). Thus it is in the interest of the animal to maintain the cuticle clear of fouling species. The mechanism(s) responsible for keeping the cuticle clean in an environment liberally stocked with fouling species have not been identified. One likely candidate is a mucous secretion from the hypodermal glands, a system of glands that discharge onto the surface of the cuticle (2). Here we show that this secretion has antibiological activities that may contribute to its ability to deter the colonization of the cuticle of Limulus by fouling organisms.

The cuticular secretion can be provoked by housing healthy adults in the presence of decaying fish (3). The secretion from the hypodermal glands was scraped from the dorsal cuticle of the Limulus shell with a rubber scraper and stored at 4°C in the presence of 0.2 mg/ml NaN₃ to prevent bacterial contamination.

Antibodies to coagulogen failed to immunostain dot blots of cuticular secretion. The anti-biological activity of the cuticular secretion was tested by its cytolytic actions on target cells. Cuticular secretion hemolysed sheep red blood cells at a 1:16 dilution in a standard hemolysis assay (7) (Fig. 1A). Hemolysis was judged to be divalent cation-dependent because the divalent cation chelator, ethylenediaminetetraacetic acid, reduced hemolysis (Fig. 1A). The macromolecular osmolytes dextran-8 (Mr 8–12 kDa) and, to a lesser extent, dextran-4 (Mr 4–6 kDa) reduced the extent of hemolysis (Fig. 1B). This suggests that hemolysis is the result of hydrophilic membrane channels established in the plasma membrane of the target red blood cell by the hemolytic protein of the cuticular secretion. Protection by the dextrans is suggested to result from their ability to balance the osmotic pressure across the permeabilized cell membrane, which will reduce the flow of water through the hemolytic pore and into the cell and will prevent swelling and lysis (8). It is difficult to envision ways for macromolecular osmolytes to protect the cell if the hemolytic process featured such other possible mechanisms as phospholipase action or detergent-mediated membrane reorganization.

It is proposed that the cuticular secretion is one agent that helps maintain the cleanliness of the cuticle of Limulus. Its

Figure 1. Hemolytic activity of the cuticular secretion from Limulus polyphemus to rabbit red cells. All samples contain a 1:16 dilution of the cuticular secretion, with the indicated additions. The hemolysis buffer is 0.25 M NaCl, 0.01 M CaCl₂, 0.14 M dextrose, 0.01 M Tris, pH 7.3. Added EDTA (10 mM) reduces hemolysis (Fig. 1A). Dextran-8 and dextran-4 reduce hemolysis (Fig. 1B).
anti-biological activity, exemplified by its ability to lyse foreign cells such as mammalian erythrocytes, may contribute to this activity. Under normal conditions, the volume of cuticular secretion is low, but it can be detected by immunological assays. Under conditions of challenge by a polluted environment, the volume of the secretion is augmented. In addition to its anti-biological activity, the continuous production of the cuticular secretion can be expected to exert a mechanical action, entrapping and sweeping potential fouling organisms away from the solid surface of the cuticle. Contrary to previous suggestions (2), the cuticular secretion does not contain secretion products of the blood cells.

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**Association of α-2-Macroglobulin with the Coagulin Clot in the American Horseshoe Crab, *Limulus polyphemus*: A Potential Role in Stabilization from Proteolysis**

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The blood clot of the horseshoe crab is established by the proteolytic conversion of the soluble clotting protein, coagulogen, into the form (coagulin) that polymerizes to establish a fibrillar extracellular clot (1). The coagulin clot functions in hemostasis (2), wound repair (3), and entrapment of bacteria invading through wounds in the cuticle. Bacteria entrapped in the coagulin clot are so strongly held that they fail even to show Brownian motion (2). Entrapment reduces the systemic dissemination of invading bacteria to locations far from the wound and its coagulin clot.

Secreted proteases serve important roles in parasitic virulence, and protease inhibitors of the plasma and blood cells of the host play a substantial role in immunity by the inactivation and clearance of the foreign proteases of invading parasites (4). In the present context, if entrapped bacteria were able to proteolyze the coagulin clot, then the period of entrapment would be reduced and the systemic dissemination of the invading bacteria would be facilitated. The sole protease inhibitor of the plasma of the horseshoe crab is α₂-macroglobulin (α₂M) (5). The blood cells also secrete α₂M when activated (6). Here we investigate the potential role of α₂M in the protection of the coagulin clot from proteolysis.

*Limulus* α₂M was purified as described previously (7). Coagulogen was purified as described by Srimal (8). Antibodies to *Limulus* α₂M and coagulogen were produced in rabbits. Western and dot blotting were conducted using standard methods and controls (9). Specimens for immunohistochemistry were fixed in 4% paraformaldehyde in seawater, quenched in 0.1 M glycine, blocked with 5% serum albumin, and stained with rabbit first antibodies and fluorescein-conjugated goat anti-rabbit IgG. Affinity resins were prepared by coupling purified protein to CNBr-activated Sepharose 4B using standard methods (10). Extracts subjected to affinity chromatography were first exposed to large volumes of plain Sepharose and were then exposed to Sepharose conjugated to the desired target protein, washed with high-salt buffer (containing 0.5 M NaCl, 10 mM Ca²⁺, 10 mM Tris, pH 7.3), and finally eluted with a buffer containing the Ca²⁺ chelator, ethylenediaminetetraacetic acid (EDTA).

Biochemical evidence for the specific binding of coagulogen to *Limulus* α₂M was provided by our ability to isolate coagulogen from detergent extracts of *Limulus* blood cells by affinity chromatography over *Limulus* α₂M-Sepharose. The protein that was eluted following removal of Ca²⁺ was identified as coagulogen by molecular mass (~21 kDa) and reactivity with anti-coagulogen antibodies (Western blotting and dot blotting). Coagulogen was by far the most abundant protein eluted from the α₂M affinity column.

Immunohistochemical and biochemical approaches were used to investigate the binding of α₂M to the coagulin clot. Blood clots were prepared *in vitro* by collecting 0.3 ml of whole blood into 90-mm petri dishes containing 10 ml of sterile, cell-free *Limulus* plasma. The cells that attach to the surface of the petri dish flatten and degranulate, and the coagulogen that is released from the secretory granules polymerizes into a fibrillar coagulin clot situated above the cell layer (11). These fibrils immunostain specifically with antisera directed against coagulogen (Fig. 1A), showing their similarity to the fibrils of the blood clot that forms *in situ* at the locations of wounds to the cuticle of the animal. When treated with an affinity-purified antibody to *Limulus* α₂M, the clot fibrils also stain brightly for *Limulus* α₂M (Fig. 1B). Treatment of the clot with 50 mM EDTA prior to fixation and processing for immunohistochemistry reduced but did not eliminate staining with

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anti-Limulus \(\alpha_2\)M antibodies (Fig. 1C). A similar pattern of decoration of clot fibrils by \(\alpha_2\)M was found in clots extracted with 0.5% Triton X-100. Control specimens treated with non-immune rabbit IgG failed to show staining of the fibrillar elements of the coagulin clot (Fig. 1D). This indicates that \(\alpha_2\)M does indeed decorate the coagulin fibrils of the clot and that the binding is largely \(\text{Ca}^{2+}\)-dependent. It is possible that the EDTA-insensitive \(\alpha_2\)M is covalently linked via transglutaminase-catalyzed isopeptide bonds. The Limulus clot does contain such protein-protein bonds (12) and human \(\alpha_2\)M is a substrate for transglutaminase crosslinking.

Additional evidence for \(\alpha_2\)M binding was provided by the characterization of the proteins that co-purify with the blood clot. For this, the clots formed in vitro were washed exhaustively with high-salt buffers (0.5 M NaCl, 10 mM CaCl\(_2\), 10 mM Tris, pH 7.3) and detergent (0.5% Triton X-100), and were then scraped from the petri dish and transferred to microcentrifuge tubes, where they were compressed by centrifugation, washed further, and finally eluted by removal of \(\text{Ca}^{2+}\) with 0.1 M EDTA. The coagulin clot was not solubilized by this treatment. The supernatant fraction contained substantial quantities of \(\alpha_2\)M, as shown by Western blotting and dot blotting using an affinity-purified antibody to Limulus \(\alpha_2\)M.

This study provides evidence that \(\alpha_2\)M binds selectively to coagulogen and coagulin in a \(\text{Ca}^{2+}\)-dependent fashion. We suggest that this serves the important function of positioning \(\alpha_2\)M from the plasma onto the fibrils of the blood clot, where it is perfectly situated to protect the clot from microbial proteolysis and the

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**Figure 1.** Immunofluorescent demonstration of the decoration of the coagulin fibrils of the blood clot by Limulus \(\alpha_2\)M. Blood cells were allowed to attach to microscope coverglasses in numbers sufficient to produce a confluent monolayer. The attached cells degranulate and produce a fibrillar coagulin clot that lies above the cell layer and stains with antibodies against coagulin (panel A, top left). The clot stains intensely with an affinity-purified antibody against Limulus \(\alpha_2\)M (panel B, top right), indicating the presence of \(\alpha_2\)M attached to the clot fibrils. Most, but not all, of the \(\alpha_2\)M is removed by treatment with 50 mM EDTA prior to fixation (panel C, bottom left). The specimen treated with non-immune serum failed to stain (panel D, bottom right). Photomicrographs B, C, and D were exposed identically to permit comparison of differing fluorescent intensities. Although most of the blood cells attach to the glass and degranulate, a few cells migrate to the top of the clot. These fail to degranulate and show as cells with autofluorescent granules in all pictures. Scale bar (Fig. 1D) = 50 \(\mu\)m.
consequent compromise of the clot’s function as a device that immobilizes entrapped bacteria.

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Enzymatic Biosynthesis of N-Linked Glycan by the Marine Sponge Microciona prolifera
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Complex carbohydrates occur in and on practically all cell types and in cellular secretions. They participate in a multitude of functions, such as fertilization, development and differentiation, cell sorting, motility, signal transduction and phagocytosis (1, 2). The Microciona aggregation factor (MAF) is a highly glycosylated compound that is involved in such functions. Glycosylation of carbohydrates proceeds enzymatically by the stepwise addition of single activated sugars to a growing sugar chain. The products may consist entirely of sugars, i.e., polysaccharides, or they may be linked to lipids or proteins. There are two types of protein-sugar binding structures: O-linked serine or threonine and N-linked asparagine. Favored N-glycosylation sites possess a characteristic binding structure: O-linked serine or threonine and N-linked glucosamine (UDP-14C-GlcNAc), with a specific activity of 7.1 mCi/nmol. A synthetic compound, trimannosyl octyl, which has proved useful as an alternate to natural substrates in which the octyl group is replaced by two GlcNAc residues (9). The co-substrate and active glycosyl donor was a 14C-labeled uridine diphosphate N-acetylglucosamine (UDP-14C-GlcNAc), with a specific activity of 7.1 mCi and 7402 dpm/nmol. Replicate tubes containing these compounds were added to a buffered cocktail that consisted of 25 mM adenosine monophosphate, 0.5 mM creatine phosphate, 0.6 mg/ml creatine kinase and buffered in 100 mM Tris at pH 7.4. The substrate was a synthetic compound, trimannosyl octyl, which has proved useful as an alternate to natural substrates in which the octyl group is replaced by two GlcNAc residues (9). The co-substrate and active glycosyl donor was a 14C-labeled uridine diphosphate N-acetylglucosamine (UDP-14C-GlcNAc), with a specific activity of 7.1 mCi and 7402 dpm/nmol. Replicate tubes containing these compounds were added to a buffered cocktail that consisted of 25 mM adenosine monophosphate, 0.5 M N-acetylglucosamine, 0.2% Triton X-100, bovine serum albumin 2 mg/ml, 40 mM MnCl₂, and 0.2 M 2-(N-morpholino) ethanesulfonic acid pH 6.1 in a total volume of 62.5 μl. After incubation for 1 h at 37°C, the reaction was terminated by the addition of 20 μl of 2% sodium borate in 20 mM EDTA. Separation of unreacted UDP-14C-GlcNAc from glycosylated radioactive product was accomplished by passing the reaction mixture through an AG1-X8 (BioRad) ion exchange column. Additional purification of the reaction product could be achieved by HPLC with C18 matrix that binds the hydrophobic product. Following a wash with deionized water, the purified radioactive product was eluted with methanol and the eluate was placed in vials for determination of counts. The average figures for product yield, expressed as nanomoles per hour per milligram of protein (nm/h/mg), were derived from duplicate counts from which were subtracted nanomoles present in endogenous assays lacking acceptor.

In a series of assays to determine acceptor-product ratios, the amount of trimannosyl substrate was increased over a fourfold range. A straight line relationship was demonstrated over a quantity of acceptor ranging from 2.5 to 10.0 μl (12.5 to 50 nmol) (Fig.

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1b). This relationship established GlcNAc-trimannosyl octyl as the presumed product catalyzed by sponge lysate GnT I activity. The specificity of this synthetic acceptor substrate has been confirmed using a wide range of Gn-T I compounds, including cell lysate from vertebrate and non-vertebrate sources, as well as the cloned and expressed rabbit, human, and mouse Gn-T I gene product (9). Final confirmation of the GlcNAc-trimannosyl link requires additional detailed NMR and spectroscopic analysis of scaled-up purified product.

The finding of a GnT I activity in the sponge raises the prospect of manipulating sponge N-glycan structures by the use of specific GlcNAc enzyme inhibitors. Gray cells, regarded as the immunocytes of the sponge (10), bear the highly N-glycosylated CD44 surface antigen (T. Simpson and W. Kuhns, unpub. data) that may be an appropriate substrate for such chemical interventions. CD44 is likely to be involved in allograft rejection events (11), and if so, alterations in its structure by de-N-glycosylation may play a role as biological response modifiers. In summary, GnT I enzyme activity appears to function in a manner very much like its counterpart in higher species. Since the sponges are the most ancient eukaryotes with a multicellular lineage, the occurrence of cellular GnT I speaks to its importance in cell functions and to its remarkable conservation over time.

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A Ca$^{2+}$-Independent Cytolytic System from the Blood of the Marine Snail, *Busycon canaliculum*

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The immune system is the ensemble of cell-based and humoral agents that protect the organism against parasites that have gained access to the internal milieu. One of the important immune activities for a variety of animals is the outright lysis of foreign cells that contact the blood (1). Cytolytic systems are frequently explored as if they were hemolytic systems, where the foreign cell that is destroyed is the mammalian erythrocyte. The red cell is easily quantified by the release of hemoglobin into the bathing medium. Here we report the presence of a hemolytic system in the plasma of the marine snail, *Busycon*.

Blood was obtained as follows. The foot of the adult whelk was wounded, and the blood flowing from the wound was collected and then immediately centrifuged to remove the cells. Hemocyanin was removed by ultracentrifugation, and the supernatant was dialyzed into Tris-buffered saline (0.15 M NaCl, 50 mM Tris, pH 7.3). Hemolysis was assayed with rabbit red cells as described previously (2).

The hemolytic activity of hemocyanin-depleted *Busycon* plasma is shown in Figure 1. Hemolysis is progressive, requiring 4 h for completion. The hemolytic activity is unaffected by the inclusion of EDTA in the hemolysis assay, and thus is independent of divalent cations. The lack of a Ca$^{2+}$ dependence distinguishes this hemolytic system from one reported previously in *Busycon* (3). Hemolysis is reduced at low ionic strength, showing a broad activity maximum in buffers containing in excess of 0.2 M NaCl. The hemolytic activity is inactivated by trypsin treatment of the plasma, indicating a proteinaceous character to the hemolytic agent to this important signature molecule of the plasma. Figure 1B, sensitivity of hemolysis to lipopolysaccharide (LPS) from the cell wall of gram-negative bacteria (Fig. 1B). The reduction in the hemolytic action at LPS concentrations sufficient to balance the osmotic pressure of hemoglobin in the cell, would protect the cell from osmotic rupture (5). The molecular size of dextran-4 is approximately 1.7 nm (6), indicating an effective pore size for the membrane-associated hemolytic protein as no larger than this value.

The hemolytic system of *Busycon* is sensitive to the presence of lipopolysaccharide (LPS) from the cell wall of gram-negative bacteria (Fig. 1B). The reduction in the hemolytic action at higher concentrations of LPS may derive from the binding of the hemolytic agent to this important signature molecule of the gram-negative bacterium, reflective of an anti-bacterial action.

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of the hemolytic agent. Although the concentrations of LPS used for Figure 1B are high, the actual concentration of LPS at the surface of the bacterium is far higher than the solution concentrations used in this trial. The possibility that the hemolytic agent does bind to gram-negative bacteria deserves further investigation.

The ability to destroy foreign cells that come in contact with the blood is an important defense strategy for a variety of animals. In mammals, the cytolyis of foreign cells is conducted by the complement system, a multi-component ensemble of plasma proteins whose membrane attack elements are activated by a proteolytic cascade that, itself, is initiated by a variety of stimuli indicative of parasitic invasion (7). The complement system is found only in the deurostomate animals (i.e., the echniderms and the chordates) and is absent from protostomate animals (8, 9). In the latter, the relatively few cytolytic systems that have been characterized are less complex than the vertebrate complement system, with some relatively few cytolytic systems that have been characterized are less complex than the vertebrate complement system, with some dependencies on the developmental stage at the time of treatment.

Modulation of the Development of Plutei by Nitric Oxide in the Sea Urchin Arbacia Punctulata

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Nitric oxide, a reactive free radical, has recently been identified as a key mediator of intercellular signaling in numerous species (1). It is produced enzymatically from l-arginine by the nitric oxide synthase family of oxidoreductases (2). Nitric oxide regulates a variety of physiological functions including relaxation of vascular smooth muscle, long-term potentiation, tumor cell apoptosis, and cytostasis (3). In addition, inappropriate or excessive production of nitric oxide has been implicated in tissue injury (4). Nitric oxide is known to initiate biochemical effects through binding to iron and iron-sulfur-containing proteins and modulating their activity (5). Nitric oxide can also modify DNA and is known to alter growth factor-mediated transcription processes (5). At the present time, no clear role for this free radical species in regulating development has been defined.

We have previously reported that nitric oxide synthase inhibitors alter fertilization and differentiation of sea urchin eggs (6). In the present studies, we examined the direct effects of nitric oxide on the development of the sea urchin Arbacia punctulata. For these studies, fertilized eggs were prepared from sea urchins, as described by Hinegardner (7), and maintained at 24°C. Embryos were exposed to S-nitroso-N-acetylpenicillamine (SNAP, Molecular Probes, Eugene, OR), an agent that spontaneously releases nitric oxide, for 1 h at various developmental stages. These stages included: immediately after fertilization, following the first and fifth divisions, the morula and prism stages, and at two points in pluteal development, i.e., 24 and 48 h after fertilization. Treated embryos and untreated controls were further evaluated at regular intervals for 72 h. Both morphological abnormalities and transient delays in development were observed, but particular effects were dependent on the developmental stage at the time of treatment. Morphological abnormalities were quantified at the morula, prism, and pluteus stages.

In initial experiments, we determined the effects of a range of concentrations of SNAP (2 nM–2 μM) applied to sea urchin eggs immediately after fertilization. Embryo mortality with little cell division was observed when embryos were treated with concentrations of SNAP in excess of 200 nM. However, both transient and permanent developmental changes were found after treatment with lower concentrations of the nitric oxide releasing agent (see further below). For our studies, we used 20 nM SNAP; at this concentration, a clear role for this free radical species in regulating development was observed. The complement system is found only in the deurostomate animals (i.e., the echinoderms and the chordates) and is absent from protostomate animals (8, 9). In the latter, the relatively few cytolytic systems that have been characterized are less complex than the vertebrate complement system, with some dependencies on the developmental stage at the time of treatment.

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Literature Cited


Developmental lesions are often induced by temporally restricted biochemical changes that eliminate critical downstream events or cause them to occur inappropriately. The resulting aberrant development reflects a stage-specific sensitivity to a pharmacological agent or event. To determine the developmental stages at which sea urchin embryos are sensitive to nitric oxide, we next exposed fertilized eggs to SNAP during various stages of development. Following fertilization, sea urchin eggs divide synchronously until the fifth division. At this stage, the embryo is designated a morula, and the uniformly distributed pigment granules become unevenly distributed to specific cells, with most going to the 8 macromere cells. When SNAP was applied after fertilization or first division, we observed no morphological alterations in the morula. Both drug-treated embryos and controls contained 2–4 darkly pigmented cells.

Sea urchin embryos hatch and become free-swimming shortly after the initiation of motility. Later, about 18 h after fertilization, the free-swimming sea urchin embryos become pyramidal, and enter the prism stage. When such embryos were exposed to SNAP, no anatomical aberrations were observed. However, embryos treated with SNAP at any stage prior to hatching exhibited delays in development (Table 1). Thus, 18 h after fertilization, embryos treated with SNAP prior to the morula stage had not progressed beyond the blastula stage. In addition, embryos exposed to SNAP before hatching contained fewer than the 6–8 darkly pigmented cells observed in controls.

Developing sea urchins generally progress to the pluteus by about 24 h after fertilization. At this stage, plutei that had been treated with SNAP immediately after fertilization, or the first division, exhibited gross morphological alterations. Additionally, the pigment cells aggregated and were fewer in number (Table 1; Fig. 1, compare panels A and B; other data not shown). This effect was associated with fewer animals progressing through development. Those that did develop further exhibited diminished arm extension when evaluated 48 h after fertilization (Fig. 1, compare panels C and D and not shown). As these plutei continued to develop, the poorly extended arms were often oriented 90° to the longitudinal axis rather than parallel to it. Aberrant morphology was less pronounced in embryos exposed to nitric oxide after division 5. In addition, when evaluated 24 h after fertilization, the number of pigment cells remained unchanged in embryos treated with SNAP before division 5, while about 4 times as many darkly pigmented cells were identified in untreated embryos.

When observed 24 h after fertilization, delayed development was again evident in embryos treated with SNAP at all stages prior to hatching. However, following exposure to SNAP at the morula stage, embryos appeared normal in development by 48–72 h after fertilization. No effects on morphology or development were observed in embryos exposed to nitric oxide at the prism stage or as plutei either 24 or 48 h after fertilization (not shown). Anatomical changes resulting from exposure to nitric oxide were preceded by an apparent inhibition of pigment cell division. These results indicate that sea urchin embryos exposed to nitric oxide before their development into morula show permanent morphological changes, whereas exposure at later stages has no apparent permanent effects.

Taken together, our results demonstrate that brief exposure of sea urchin embryos to nitric oxide during early development causes irreversible abnormalities in plutei, including skeletal

### Table 1

<table>
<thead>
<tr>
<th>Developmental stage at fertilization (embryos/50 µl)</th>
<th>Morula</th>
<th>Prism</th>
<th>Pluteus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1 ± 2.3</td>
<td>0 ± 0.6</td>
<td>13 ± 3.9</td>
</tr>
<tr>
<td>Following fertilization¹</td>
<td>24 ± 6.1</td>
<td>7 ± 3.2</td>
<td>0 ± 0.3</td>
</tr>
<tr>
<td>First Division</td>
<td>27 ± 6.0</td>
<td>14 ± 2.3</td>
<td>1 ± 3.3</td>
</tr>
<tr>
<td>Division 5</td>
<td>19 ± 4.0</td>
<td>10 ± 1.9</td>
<td>0 ± 3.4</td>
</tr>
<tr>
<td>Morula</td>
<td>1 ± 4.3</td>
<td>4 ± 0.8</td>
<td>9 ± 2.3</td>
</tr>
<tr>
<td>Prism</td>
<td>0 ± 3.4</td>
<td>0 ± 0.9</td>
<td>21 ± 4.5</td>
</tr>
<tr>
<td>Pluteus</td>
<td>2 ± 3.3</td>
<td>1 ± 0.3</td>
<td>13 ± 2.6</td>
</tr>
</tbody>
</table>

¹ Approximately 10⁴ embryos were suspended in 10 ml of sea water supplemented with 20 nM SNAP at the indicated developmental stages. After 1 h, embryos were transferred into 100 ml of SNAP-free sea water. Embryos were then evaluated for progress through development at 24 h after fertilization. Each point represents the average number of embryos in each stage of development for 5 experiments ± SEM.

Figure 1. Effect of nitric oxide on the development of sea urchin eggs. Immediately following fertilization, sea urchin eggs were treated with 20 nM SNAP. After 1 h the embryos were washed and allowed to develop. Panels A and B are sea urchin embryos 24 h after fertilization; panels C and D show embryos 48 h after fertilization. Panels A and C are control embryos; panels B and D are embryos that have been treated with SNAP.
aberrations and changes in the proliferation and migration of pigment cells. The effects of nitric oxide applied at later stages are reversible. We speculate that nitric oxide may act as a negative regulator of pigment cell division and skeletal extension in vivo.

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Development of Self-Referencing Oxygen Microsensor and its Application to Single Pancreatic HIT Cells: Effects of Adenylate Cyclase Activator Forskolin on Oxygen Consumption
Sung-Kwon Jung*, Katherine Hammar, and Peter J. S. Smith (BioCurrents Research Center, Marine Biological Laboratory, Woods Hole, Massachusetts 02543)

Information about oxygen dynamics is valuable for evaluating the metabolic activity of pancreatic β cells, which contribute to the regulation of glucose homeostasis in the blood. When glucose, the key secretagogue, is metabolized in β cells, intracellular messengers such as ATP are formed. ATP-sensitive potassium channels (KATP) then close, which depolarizes the cell, leading to Ca2+ entry and stimulation of insulin secretion by exocytosis (1, 2). The relationships between metabolism, oxygen consumption, insulin secretion, and other biochemical changes in pancreatic islets have been examined (3–5). In the present investigation, we focused on the relationship between altered cAMP levels and oxygen consumption. According to Ammala et al., insulin release by intracellular cAMP is calcium-independent (6). But exocytosis is metabolically driven, and intracellular calcium levels are tightly correlated with oxygen consumption (3). Therefore, we need to study the oxygen dynamics of β cells, but at the level of a single cell, because pancreatic islets are composed of four distinct cell types. However, such studies with single β cells are very difficult, primarily because single cells generate very small changes in oxygen level during respiration. Often, therefore, the electrochemical drift of an oxygen sensor dwarfs such small changes, so interpretation of the data is difficult. We have now developed a self-referencing oxygen microsensor with a tip diameter < 3 μm and have measured the oxygen consumption of single pancreatic HIT cells.

The self-referencing technique has been successfully used to measure selected ion and oxygen fluxes at single cell levels by minimizing the impact of sensor drift and noise (7, 8). The technique is based on the translational movements of a microprobe between two points in the concentration gradient that extends outward from the cell membrane. The difference in the values measured at two points provides information about the gradient. The oxygen sensor used in the current study was designed to have the characteristics required for the self-referencing technique, such as minimal convection perturbation and a response time < 1 s. The sensor has an etched Pt wire electrochemically recessed inside a pulled glass micropipette, which was coated with cellulose acetate (9).

To validate the applicability of the self-referencing oxygen sensor to flux measurements, an oxygen gradient was artificially generated and measured (Fig. 1A). The apparent oscillation of the DC trace is the result of sensor relocations in the oxygen gradient. The translational frequency was optimized for maximal sensitivity (0.2 Hz). For example, as observed in Figure 1A, the signal rapidly equilibrated as the sensor was repeatedly brought to a new position in the self-referencing mode compared to the stationary mode. IonView (a product of BioCurrents Research Center, Marine Biological Laboratory, Woods Hole, MA) automatically subtracts current values at the far position from those at the near position. The first half of the values at each position were discarded to eliminate the artifacts from sensor motion and response time; the second half were sampled for mathematical subtraction (7). The resulting data, designated as AC, are used for calculating oxygen flux according to the Fick equation:

$$ J = -D_o \frac{\Delta C}{\Delta r}, \quad (1) $$

where J is flux (mol cm$^{-2}$ s$^{-1}$), $D_o$ is the diffusion coefficient of oxygen (2.6 × 10$^{-5}$ cm$^2$ s$^{-1}$), and $(\Delta C/\Delta r)$ is the concentration difference divided by the translational distance (i.e., the concentration gradient).

We applied the self-referencing oxygen sensor to single pancreatic HIT cells. Oxygen consumption was significantly decreased upon the injection of rotenone, an inhibitor of mitochondrial respiration (Fig. 1B). Based on equation 1, the oxygen flux with a Δr of 30 μm at 25 μm from the plasma membrane of single cells was 640 ± 40 fmol/cm$^2$/s (mean ± SEM, n = 5) at 11 mM glucose, which is then converted into a consumption (3.2 fmol/cell/min) by taking the geometric surface area into account. After rotenone injection, the oxygen flux significantly decreased to 97 ± 15 fmol/cm$^2$/s (mean ± SEM, n = 5), a consumption of 0.4 fmol/
the plasma membrane of the cell and oscillated between 10 and 40 pmol/cell/min (see Fig. 1C). The oxygen consumption after forskolin treatment (10) was significantly higher than before (P < 0.01). This increase (∼19%) may account for the ATP demand in insulin secretion.

Calcium ions may have a role in the response of HIT cells to adenylyl cyclase activation. Oxygen dynamics and intracellular Ca2+ ([Ca2+]i) are well correlated in islet cells, in that increases in oxygen consumption closely follow increases in [Ca2+]i, according to the observation by Jung et al. (3). The activation of adenylyl cyclase will lead to a decrease in ATP levels unless ATP is rapidly replenished. Therefore, we postulate that the forskolin-induced increase in oxygen consumption reflects the release of Ca2+ from subcellular organelles, a notion supported by the finding that blockage of sarco-endoplasmic reticulum Ca2+-ATPase (SERCA) pumps by thapsigargin abolishes transient increase in [Ca2+]i, induced by forskolin (10). On the other hand, cAMP could directly deinhibit the activity of phosphofructokinase, a controlling enzyme of glycolysis, resulting in NADH elevation and then an increase in oxygen consumption. The above intertwined relationships could be explained by a recent report suggesting that, while Ca2+ may activate respiration, its entry into β cells has a net effect of decreasing the ATP/ADP ratio resulting from the activation of numerous ATP consuming processes, such as insulin secretion and ion-pumping (11).

In conclusion, the development of a self-referencing oxygen microsensor and its application to single HIT cells hold promise for the elucidation of the relationship between oxygen dynamics and the role of cAMP in exocytosis. Further use of the self-referencing oxygen sensor will include correlated measurements with [Ca2+]i, ATP/ADP and insulin secretion at the single cell level.

We thank Richard Sanger for technical assistance. This work was supported by the NIH Grant NCRR P41 RR01395 to PJS Smith.

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Figure 1. (A) Real time trace (DC) and IonView processed trace (AC) of oxygen current as a function of distance from an oxygen source (diameter = 10 μm) measured with a stationary and a self-referencing oxygen microsensor. In the self-referencing mode, the oxygen sensor was oscillated within a 10-μm excursion at a frequency of 0.2 Hz. The numbers are the relative distance from the oxygen source in microns. (B) Influence of rotenone on oxygen consumption by a single pancreatic HIT cell. The trace is a representative of five measurements. (C) Influence of forskolin on oxygen consumption by a single pancreatic HIT cell. The trace is a representative of four measurements. There is a significant difference in oxygen consumption after forskolin treatment (P < 0.01). In the above single cell measurements, the oxygen sensor was positioned 10 μm from the plasma membrane of the cell and oscillated between 10 and 40 μm (a 30-μm excursion) at a frequency of 0.2 Hz. A potential of −600 mV vs. Ag/AgCl was applied to the oxygen sensor in a modified HEPES buffer at 37°C for all experiments.

(cell/min (P < 0.001 according to two-tailed Student’s t tests). This decrease (∼88%) is presumably due to the inhibition of NADH-dependent respiration. We injected forskolin, a specific activator of adenylyl cyclase, to elevate cAMP level and observed a flux of 0.76 ± 0.07 pmol/cm2/s (mean ± SEM, n = 4), a consumption of 4.0 fmol/cell/min (see Fig. 1C). The oxygen consumption after forskolin treatment was significantly higher than before (P < 0.01). This increase (∼19%) may account for the ATP demand in insulin secretion.

Calcium ions may have a role in the response of HIT cells to adenylyl cyclase activation. Oxygen dynamics and intracellular Ca2+ ([Ca2+]i) are well correlated in islet cells, in that increases in oxygen consumption closely follow increases in [Ca2+]i, according to the observation by Jung et al. (3). The activation of adenyl cyclase will lead to a decrease in ATP levels unless ATP is rapidly replenished. Therefore, we postulate that the forskolin-induced increase in oxygen consumption reflects the release of Ca2+ from subcellular organelles, a notion supported by the finding that blockage of sarcoplasmic reticulum Ca2+-ATPase (SERCA) pumps by thapsigargin abolishes transient increase in [Ca2+]i, induced by forskolin (10). On the other hand, cAMP could directly deinhibit the activity of phosphofructokinase, a controlling enzyme of glycolysis, resulting in NADH elevation and then an increase in oxygen consumption. The above intertwined relationships could be explained by a recent report suggesting that, while Ca2+ may activate respiration, its entry into β cells has a net effect of decreasing the ATP/ADP ratio resulting from the activation of numerous ATP consuming processes, such as insulin secretion and ion-pumping (11).

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Literature Cited
The origin of hemocytes, the circulating “blood cells” of bivalve molluscs, including hard clams (Mercenaria mercenaria) has not been identified (1, 2). Proliferation of hemocytes, however, can be recognized through their increased numbers in diseased animals. Proliferating cell nuclear antigen (PCNA), or cyclin, is a protein produced during the late G1 and S phases of the cell cycle (3, 4). Using antibodies that recognize PCNA in mice, we attempted to identify the origin of hemocytes in the hard shell clams. Quahog parasite unknown (QPX) is a protist that causes severe inflammation and mortality in infected clams (5, 6). We attempted to induce hemocyte proliferation by exposing clams to QPX in a 10-l water column in which 12 ml of undiluted QPX culture (at a concentration of $7 \times 10^6$ cells/ml) were added every 10 days; by injecting QPX between the membranous mantles and the right valves, 3 cm ventral anterior to the siphon and into the pericardial cavities (0.25 ml of undiluted QPX culture); and by injecting an inert particle (India ink, 1:10 dilution in sterile seawater [7, 8]) into the pericardial cavities. The controls consisted of two groups of clams. One group was injected with sterile seawater in the pericardial cavities; the other was untreated. Groups were sampled at 24 h, and at 1, 4, and 8 weeks after the start of the experiment. At sampling, the animals were shucked, fixed in 10% neutral buffered formalin (NBF) for 24 h, and embedded in paraffin. Sections were cut (4–6 μm), mounted onto positively charged slides (Fisher-brand, Superfrost/Plus and ProbeOn Plus slides) and stained either with hematoxylin and eosin (H&E) (9) or with anti-PCNA with a hematoxylin counterstain (Zymed, PCNA Staining Kit).

Clams injected with QPX in the pericardial cavities showed mild focal inflammation associated with viable and necrotic QPX organisms. At 2 months post-injection, viable QPX organisms were no longer identified. QPX organisms and associated inflammation were not observed in clams injected in the mantle cavity. After 2 months of water column exposure, only very rare infection by QPX organs with minimal inflammation was observed in mantle tissue. India ink injection caused a minimal inflammatory response. Pools of injected ink in the tissues and vascular spaces were either engulfed by individual hemocytes or surrounded and sequestered by hemocytes (encapsulation), forming thin-walled granulomas (6, 10). Numerous individual hemocytes containing India ink were eliminated from the clams by diapedesis over luminal epithelial surfaces (Fig. 1A). Thick-walled granulomas (6, 10) were also identified in the gills, pericardial sacs, and other

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Figure 1. Proliferating epithelial cells and hemocytes of Mercenaria mercenaria stained with anti-Mouse Proliferating Cell Nuclear Antigen (PCNA). (A) India ink is phagocytized and eliminated by diapedesis of India ink-filled hemocytes (arrow) into the renal tubular lumens (H&E, bar = 13 μm). (B) Nuclei of proliferating reserve cells of the digestive gland’s tubular epithelia stained black (arrow) with anti-PCNA. PCNA negative nuclei stain blue (arrowhead) (hematoxylin counterstain, bar = 13 μm). (C) Strong PCNA nuclear staining is present in the proliferating nuclear epithelial cells at the base of the gills (arrows). PCNA negative nuclei stain blue (arrowhead) (hematoxylin counterstain, bar = 13 μm). (D) Nuclei of some hemocytes in thick-walled granulomas in the kidney tissue of clams are positive for PCNA stain (arrows) and present evidence for the proliferation of hemocytes directly at the inflammatory site (hematoxylin counterstain, bar = 13 μm).
ConditionS Affecting the Growth and Zoosporulation of the Protistan Parasite QPX in Culture

Christine Brothers¹, Ernest Marks III², and Roxanna Smolowitz

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Quahog Parasite Unknown (QPX) is a protistan disease of hard clams (Mercenaria mercenaria). The QPX organism has been classified in the phylum Labyrinthomorpha (1, 2). Disease resulting from QPX infection has been identified in New Brunswick and Prince Edward Island, Canada; Barnegat Bay, New Jersey; Chatham, Duxbury, and Provincetown, Massachusetts; and three locations in Virginia (1, 2). Mortality from QPX can be severe, with losses especially high in clams just under market size (about 2 years old). An important clinical sign of infection is the occurrence of QPX-infected inflammatory nodules in the mantle.

Whyte et al. (3) isolated QPX cells from infected clams; when placed in artificial seawater, these cells produced sporangia and zoospores. Kleinschuster and Smolowitz (2) recently described continuous in vitro culture of QPX. QPX was isolated from inflamed mantle nodules and cultured in modified MEM medium at pH 7.2 at 22°C. Mature cultures (after 5 to 10 days) showed thalli, immature sporangia, and mature sporangia containing endospores. Organisms in these stages ranged from 5 to 120 μm in diameter.

Endospores released from mature sporangia became the new thalli. Cultured QPX organisms produced, and were embedded in, a thick mucoid material that could be removed intact from the remaining unused culture medium. When placed into sterile seawater, QPX produced motile zoospores within 4 days.

The effects of different environmental conditions on the occurrence of QPX and the resulting disease in the field are unknown. Determination of how environmental parameters affect cultured QPX may help in understanding the pathogenesis of the disease in the field. In this study, the environmental effects of temperature, pH, and salinity were investigated on QPX cells culture.

Medium (pH 7.2 and salinity 40 ppt) was prepared using the standard methods (2). Modified medium (40 ppt) was prepared at pH 6.0, 7.0, and 8.0 by adjusting pH with 2 M HCl and 2 M NaOH. Modified medium (pH 7.2) was also prepared at 20, 28, and 34 ppt by proportionally reducing the salt content of the medium and monitoring the resulting solutions with a refractometer. All media were filter sterilized. To test the effects of pH and salinity on the proliferation of QPX in culture, 0.4 ml of two QPX subcultures was placed in a culture flask with 10 ml of each of the three pH variations or four (including standard) salinity variations. Fourteen cultures were created (seven of each subculture). These cultures were incubated for 10 days at 22°C. To test the effects of temperature on QPX growth in culture, the same procedure was followed.

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using 10 ml of standard medium to create a total of 12 cultures. One flask of each subculture was incubated at six temperatures (0°, 10°, 16°, 22°, 32°, 38°C) for 10 days.

Concentrations of the mucus containing QPX from each of the four initial subcultures, as well as from the 26 experimental cultures at the end of the 10-day incubation, were determined. The QPX-containing mucus was extracted from the culture medium and measured using a 10-ml pipette. Concentrations of QPX organisms were measured by counting the number of cells per milliliter of a 1:10 saline dilution of the mucus, using a hemocytometer. Initial concentrations of QPX averaged 1.4 million cells per milliliter (range = 1.1 to 1.8 million). Final concentrations and volumes are shown in Figure 1.

QPX concentrations per milliliter and volume of mucus produced both increased with increasing pH and increasing salinity. In culture, the modified MEM medium becomes more acidic as the culture matures. This lower pH may inhibit further growth of the culture. At low salinities, QPX thalli have previously been observed lysing, which may explain the lower cell concentrations at 20 ppt. The cell concentration was highest at 0°C and decreased with increasing temperature. This may represent a thinning out and spreading of the mucus containing the cells with increasing temperature. However, the total number of QPX organisms and total mucus production was greatest at 24°C. The volume of mucus containing QPX produced was low from 0° to 16°C, peaked at 24°C, then declined with increasing temperature. Above 32°C, there was no growth of QPX and no mucus production. Whether QPX will grow above pH 8.0 and above 40 ppt should be investigated.

Proliferation of cultured QPX is best at a temperature of 24°C, pH 7 to 8, and salinities of 28 ppt and above. Such findings are consistent with the field observation of increased infection in the summer and occurrence of QPX disease primarily in high-salinity waters.

Conditions affecting the zoosporulation of QPX were also investigated. Seawater (pH 8.0, salinity 30 ppt) was adjusted to pHs of 6.0, 7.0, and 9.0 (using 2 M HCl and 2 M NaOH) and to salinities of 20 and 40 ppt (by dilution with distilled water or addition of NaCl), then filter sterilized. Concentrations of 1% and 10% QPX in seawater of each pH and salinity were placed in replicates in a 24-well sterile plate. The plates were incubated at 10°, 16°, 22°, 32°, and 38°C and examined daily for 5 days. Other researchers (2, 3) have reported zoosporulation in QPX; however, there is reason to believe these may not have been from pure cultures of QPX. This study attempted to replicate those findings; however, no zoospores were observed in repeated trials, even in normal sterile seawater. Whether in fact QPX produces zoospores, as not all members of the phylum do, and under what conditions it does so are important both in the further classification of the organism and in studying transmission of the disease. Therefore, this question deserves further investigation.

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The movement of vesicles and organelles in cells has been shown to occur on both microtubules and actin filaments (1, 2). The transport of vesicles on microtubules permits movement over long distances in cells, while the short-range transport of vesicles on actin filaments produces the fine and precise movements required to position vesicles at membrane sites for capture, docking, or fusion (3, 4). The transport of vesicles on actin filaments requires vesicle-associated myosins, some of which have been identified. Myosin V, for example, transports endoplasmic reticulum (ER) vesicles (5–7) and synaptic vesicles (8) in neurons and is involved in the capture of melanosomes at the tips of mouse melanocytes (9). In addition, myosin V is involved in the transport of lysosomes in some cells (10), secretory vesicles in yeast (11), and melanosomes in Xenopus melanophores (12).

Myosin II, also known as conventional myosin, is vesicle-associated in extracts of clam (Spisula solidissima) oocytes (4, 13). In addition, myosin II was reported to be associated with Golgi vesicles (14), but the antibody used in these studies cross-reacts with coatomer proteins on Golgi-derived vesicles (15–17). Therefore, the role of myosin II in vesicle transport remains controversial. In this study we performed antibody-inhibition experiments with extracts of clam oocytes to provide additional evidence that myosin II is a vesicle motor. Clam oocyte extracts are well characterized and have been used extensively to study cell cycle events including cyclin synthesis and degradation, as well as ubiquitin-mediated proteolysis of cyclins (18).

Extracts were prepared from quiescent oocytes by the method of Ruderman et al., (18) with slight modifications. Fluorescence microscopy was used to view rhodamine-phalloidin stained actin filaments in the extracts, and AVEC-DIC microscopy was used to assay vesicle transport on actin filaments. Motile activity in extracts was determined by counting the number of vesicles moving per minute per field (v/m/f). Antibody-inhibition experiments were performed with an antibody made to oocyte myosin II. The myosin II in oocyte extracts was precipitated with ammonium sulfate, dissolved in buffer (20 mM Tris pH 7.2, 1 mM EDTA-K, 0.2 mM CaCl2, 1 mM DTT), and dialyzed overnight at 4°C. The pure fraction of the myosin II was prepared by running the solubilized fractions on SDS-gels and eluting the myosin II heavy chain band. The eluted protein was used for antibody production in rabbit. The serum of the immunized rabbit was collected, and the polyclonal antibody (ap-205) was purified on protein A beads.

The antibody (ap-205) recognized a single band at 205 kD on western blots of oocyte extracts (Fig. 1A) and in myosin II-enriched fractions (Fig. 1B). The protein recognized by (ap-205) was also recognized by a myosin II antibody, M2.42 (Fig. 1A), produced to a peptide in the head domain of Acanthamoeba.
myosin II (gift of Donald Kaiser). Therefore, the op-205 antibody exhibited high affinity and specificity for clam myosin II. In immuno-precipitation (IP) experiments with op-205, proteins of 205 and 45 kD, the respective molecular weights of myosin II and actin, were present in the precipitate (Fig. 1C, lane 2). A blot of the op-205 immuno-precipitate probed with op-205 (Fig. 1C, lane 3) identified the 205 kD band as myosin II, and a blot with an antibody to actin identified the 45 kD band as actin (data not shown). The 4 additional bands seen on both the gel and blot were present in the protein A-purified antibody (Fig. 1C, lane 1); therefore, they represent serum proteins rather than proteins in the oocyte extracts. The IP data provided evidence that the antibody binds to native myosin II and may serve as a function-blocking antibody. We therefore examined the effects of the op-205 antibody on actin-dependent vesicle transport.

The protein-A purified op-205 antibody was concentrated to 3 mg/ml and buffer-exchanged into vesicle motility buffer (T buffer, pH 7.2) for antibody-inhibition experiments. An antibody (oQLLQ) made to squid myosin V (5) that does not detect oocyte proteins on western blots was used as the control. Extracts were prepared for motility assays with either 0.38 or 1.0 mg/ml of op-205, and a control sample was prepared at the same time with oQLLQ. The motile activity was determined in the control and the treated samples at regular intervals for a period of 1.5 hours. Motile activity in the control remained at 412 ± 96 vesicles/minute/field (v/m/f) for the observation period, while the extracts treated with 0.38 mg/ml op-205 decreased to 232 ± 51 v/m/f (43% inhibition). At 1.0 mg/ml op-205, motile activity decreased to 199 ± 58 v/m/f, while the control remained at 363 ± 71 v/m/f (45% inhibition). These data showed that op-205 inhibited 40% to 45% of the motile activity in clam oocyte extracts. We plan to use affinity-purified op-205 to determine whether motile activity is inhibited completely.

In summary, the inhibition of vesicle transport by a myosin II-specific antibody provides evidence in support of the conclusion that myosin II in clam oocytes functions as a vesicle motor. The lack of 100% inhibition by op-205 may suggest the involvement of other myosin motors in actin-based vesicle transport in oocytes. The vesicles in these extracts are probably ER-derived, and myosin II may therefore be involved in the transport of ER vesicles during the early events of fertilization and embryonic development.

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Interaction of Actin- and Microtubule-Based Motors in Squid Axoplasm Probed with Antibodies to Myosin V and Kinesin

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Myosin V, an actin-dependent molecular motor highly expressed in neurons, transports ER vesicles on actin filaments in the giant axon of the longfin squid, Loligo pealei (1). The amino acid sequence of squid brain myosin V is similar to those of mouse and human myosin Va (2). In a recent study, Huang et al. (3) showed that the rod-tail domain of ubiquitous kinesin (aa680 to aa1100) and the AF6/cno globular tail domain of MyoV (aa1643 to aa1800) bind to each other. This led to the hypothesis that the two motors form a complex on vesicles through tail-tail interaction. The direct interaction of these motors could provide a mechanism by which vesicles move efficiently from microtubules to actin filaments, as postulated in the dual filament model of vesicle transport (4–7).

In this study, we used antibodies raised to squid brain myosin V (oQLLQ) and squid brain kinesin (H2 antibody provided by...
Dr. S. Brady) to determine whether kinesin binds to myosin V in squid neurons. In addition, we used recombinant tail fragments of mouse MyoV and mouse kinesin (constructs provided by Dr. Huang) to test whether these fragments form heterologous complexes with squid brain kinesin and myosin V, respectively. The plasmids containing the glutathione S-transferase (GST)-labeled mouse AF6/cno tail-globular-domain (GST-MyoV-tail aa1569 to aa1768 without the coiled medial tail domain) and the histidine (His)-labeled, kinesin-tail fragment of mouse ubiquitous kinesin (aa763-856) were expressed in E. coli. The bacterially expressed mouse GST- or His-tagged fragments were purified on affinity columns and used in binding assays of squid brain extracts.

The specificity of αQLLQ and H2 was determined by probing blots of squid brain extracts with the two antibodies. αQLLQ recognized a single protein of 196 kD in blots of squid brain extracts (Fig. 1A, lane 2), and H2 recognized a single protein of 120 kD (Fig. 1A, lane 3). These antibodies were then used in immunoprecipitation experiments to determine whether myosin V and kinesin interact directly. Squid brain extracts were incubated with the αQLLQ and then with protein-A beads. The precipitate was collected by centrifugation and run on a SDS-PAGE gel (Fig. 1B, lane 1). Proteins in the 50–55 kD range representing the antibody were the primary components in the precipitate. A blot of the immuno-precipitate probed with αQLLQ showed a myosin-V band at 196 kD, protein bands at 50–55 kD representing the antibody, and one unknown protein at about 100 kD. The unknown protein may represent a serum protein that binds to the antibody during protein-A purification or a breakdown product of myosin V. A blot of the immunoprecipitate was probed with H2, and a protein of 120 kD representing kinesin was revealed (Fig. 1B, lane 3). Therefore, these data support the hypothesis that kinesin is a binding partner of myosin V and the interaction may function to regulate the motor activity of myosin V.

In corollary experiments, squid brain extracts were incubated with the kinesin antibody H2 followed by incubation with protein-A beads. The precipitate was collected by centrifugation and analyzed by SDS-PAGE. Multiple bands were observed, including a protein of 120 kD and one of 200 kD (Fig. 1C, lane 1). The antibody (50 kD) was the other major band on the gel. The 120 kD protein was shown to be kinesin by probing a blot with H2 (Fig. 1C, lane 2). The H2 antibody recognized several other unknown proteins. These results complement those obtained with αQLLQ and support the conclusion that kinesin and myosin V bind to each other in neurons.

The purified mouse GST-MyoV-tail fragment and the His-labeled kinesin-tail fragment were analyzed by SDS-PAGE, transferred to nitrocellulose, and probed with αQLLQ and H2. αQLLQ and H2, respectively (data not shown), did not detect the GST-MyoV-tail fragment and the His-kinesin-tail fragment. The αQLLQ antibody was made to a 14-amino acid synthetic peptide in the AF6/cno domain of squid myosin V (8), so its failure to recognize the mouse protein fragment was not surprising. Affinity isolation experiments were performed to determine whether the GST-MyoV-tail fragment binds to squid brain kinesin. Squid brain extracts were incubated with the GST-MyoV-tail fragment and then with glutathione beads. The beads were washed 5× with PBS and eluted by the addition of 3 bead-volumes of 1× glutathione, then analyzed by SDS-PAGE. A blot of the precipitate probed with an antibody to squid brain kinesin (H2) did not show kinesin as one of the proteins in the complex (data not shown). In a similar experiment, purified mouse His-kinesin-tail fragment was analyzed for its ability to interact with squid brain myosin V. Squid brain extracts were incubated with the His-labeled kinesin fragment followed by incubation with the His-antibody. The antibody kinesin-fragment was precipitated with protein A-Sepharose beads and analyzed on SDS-gels. Blots of the immuno-precipitate probed with an antibody to squid brain myosin V (αQLLQ) did not show myosin V as one of the proteins in the complex. Therefore, these data suggested that heterologous complexes do not form between the mouse MyoV-tail and native squid kinesin and between mouse kinesin-tail and native squid myosin V.

In summary, these experiments demonstrate that squid brain myosin V and squid brain kinesin are binding partners in neurons. The mouse recombinant fragments and squid native proteins did not form complexes that could be detected on blots. Therefore we plan to produce squid recombinant proteins to determine whether vesicle transport is inhibited in axoplasm. The inhibition of vesicle transport by tail fragments of myosin V and kinesin has been shown in cells grown in culture (9, 10) but has not been demonstrated in vitro.

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Microtubule-Dependent Nuclear Positioning and Nuclear-Dependent Septum Positioning in the Fission Yeast Saccharomyces pombe

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The correct placement of the cell division plane is important for cell shape, size, and orientation, and for the proper partition of cellular determinants for development. The position of the division plane has been shown to be dependent on microtubules in many organisms (1). What molecular mechanism of the fission yeast ensures that the division plane and septum will be properly positioned?

Fission yeast is a rod-shaped cell that divides by medial cleavage. The nucleus is located at the geometric center of the cell, attached to multiple bundles of dynamic, anti-parallel microtubules that push on the nucleus (2–4; our unpublished results). The position of the division plane and septum coincides with the position of the interphase nucleus (5). By examining the effect of depolymerizing microtubules within the cell, we tested the hypothesis that microtubules dictate the central position of the nucleus; and the position of the nucleus, in turn, dictates the future position of the septum.

A wild-type fission yeast strain (h– leu1-32 nup107-GFP nmt-GFP-atb2), expressing (1) fusion of a nuclear pore protein to the green fluorescent protein and (2) fusion of the green fluorescent protein to tubulin, was used to visualize both the nucleus and the microtubules. For imaging, cells grown to mid-log phase in liquid media (EMM + 5 μg/ml thiamine) at room temperature (21°C–23°C) were mounted between a coverslip- and slide-sealed chamber containing a thin pad of 2% agarose and yeast media (YE5S). Methyl-2-benzimidazole-carbamate (MBC), a potent inhibitor of microtubule polymerization, was used from fresh stock (YE5S). Time-lapsed images (1 h-interval, 1 s-exposure time) were digitally acquired at room temperature (21°C–23°C) with Metamorph Software (Universal Imaging Corp.) controlling a CCD digital camera (Orca-1, Hamamatsu Corp.), attached to a Leica DMRX microscope stand equipped with DIC optics, as well as with a PL Fluotar 100×/1.3NA oil-immersion objective (Leica Corp.) and a mercury arc lamp for wide-field epifluorescent microscopy.

To determine whether microtubules play a role in the placement of the nucleus at the cell center, we used time-lapse microscopy to examine the position of the nucleus in interphase cells treated with the microtubule-depolymerizing drug MBC. Nuclear position in a cell can be expressed as the ratio of two lengths: the length from the center of the nucleus to the shorter cell tip (Lshort), and the length from the center of the nucleus to the opposite longer cell tip (Llong). The ratio Lshort/Llong = 1 when the nucleus is exactly at the cell geometric center, and Lshort/Llong < 1 when the nucleus is off-center. MBC-treated cells, which have no microtubules and ultimately die, continued to lengthen for several hours at a rate similar to that of control cells, ~1.5 μm/h. During a 2-h period, the control cells grew from an average length of 9.16 ± 1.04 μm to 12.16 ± 1.47 μm (N = 23 cells). Almost all nuclei were positioned in the middle of the cell, with an average Lshort/Llong ratio of 0.96 ± 0.03; and ~96% of cells had better than 0.90 ratio. In contrast, MBC-treated cells grew from an average length of 8.73 ± 1.55 μm to 11.45 ± 2.01 μm (N = 40 cells), with many offset nuclei, an average Lshort/Llong ratio of 0.80 ± 0.15; and only ~28% of cells had better than 0.90 ratio (Fig. 1A). Clearly, while MBC did not affect the cell growth rate, the proper central positioning of the nucleus was dependent on microtubules.

To test whether the position of the nucleus dictates the position of the division plane and septum, we examined the position of the septum in MBC-treated cells, which have off-center nuclei. Lacking microtubules, MBC-treated cells showed a delay in the cell cycle, and a curvilinear or “bent” growth pattern (6, 7). Whereas control cells exhibited a cell cycle time of ~4 h, the cell cycle in MBC-treated cells was significantly delayed to ~6–8 h. However, MBC-treated cells attempted to divide at the end of the cell cycle delay, and eventually each cell formed a septum at the site of the offset nucleus that “cut” the nucleus. The new daughter cells, which have off-center nuclei. Lack-


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position of the plane of cell division and the septum. Cells which have lost their microtubule cytoskeleton can continue to grow, and can undergo septation and cytokinesis after a cell cycle delay.

Our results are consistent with phenotypes seen in studies of tubulin mutants (7). However, our experimental conditions ensure an almost complete loss of the microtubule cytoskeleton; and our time-lapse microscopy allows long-term viewing of the development of phenotypes.

It has been proposed that cytokinesis factors may be localized to the nuclear region by association or movements on microtubules (8). However, our studies suggest that microtubules may not be strictly required for the assembly or localization of the ring at the nucleus.

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**Figure 1.** Cells treated with MBC exhibit a misplaced nucleus and septum. Fission yeast cells expressing nucleoporin-GFP and GFP-tubulin were imaged at room temperature (21°–23°C) with time-lapse fluorescence microscopy. (A) Plot of distribution frequency of nuclear positions expressed as the ratio $L_{\text{short}}/L_{\text{long}}$, where $L_{\text{short}}$ is the length from the center of the nucleus to the short cell tip and $L_{\text{long}}$ is the length from the center of the nucleus to the opposite long cell tip. (B) Wild-type cells through one cell cycle. Left panels are DIC images showing septum formation at the middle of the cell (at 0 h and later at 4 h). Right panels are fluorescent images of nuclear membrane and microtubules. Microtubules span the length of the cell during interphase. As the cell grew, nuclei were positioned by dynamic microtubules at the center of the cells where subsequent cell division and septation occurred, creating two daughter cells of approximately equal length. Note the disappearance of interphase microtubules in the cell cytoplasm and the appearance of the mitotic spindle inside the cell nucleus during mitosis (at 4 h). (C) MBC-treated cells through one cell cycle. No microtubules were present in MBC-treated cells. Without microtubules, the nuclei were offset, the cell cycle was delayed, no spindles were formed, and subsequent division planes and septum were also offset, creating “cut” nuclei and daughter cells of unequal length. The DIC panels from 0–4 h show a cell with a “birth scar,” not to be confused with the septum. Bar = 10 μm.
The Role of Microtubules During Blastodisc Formation of the Squid, *Loligo pealei*

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After fertilization, cytoplasm streams from the vegetal region of the squid egg towards the animal cap to form a blastodisc where meroblastic cleavage will occur (1, 2). This process begins at fertilization, accelerates after second polar body formation (90 min, at 20°C), and continues through third cleavage (6.5–7.0 h). A blastodisc cap is formed, although at a slower rate, in eggs that have been artificially activated with 10 μg/ml A23187 (Molecular Probes) (3). To explore the role of the cytoskeleton in this process, *in vitro* fertilized (4) or activated embryos were placed in small petri dishes lined with 0.2% agarose (Sigma, Type II) and filled with 20°C Millipore-filtered seawater (MFSW). The dishes were placed on ice and cooled to 4°C. Exposure to cold was chosen to perturb cytoplasmic movements targeting microtubules (5), so that the effect on the embryos could be easily reversed. Cold treatment periods were selected to include the first and second polar body meiotic divisions (20 min and 1.5 h respectively), and the first (3.5 h), second (4.0 h) and third (6.5 h) cleavage events. Treatment periods were 20 min to 3 h, 3 to 4 h, 4 to 5 h, 5 to 6 h and 6 to 7 h of development. After treatment, dishes of embryos were removed from the ice and allowed to return to room temperature (20°C).

Embryos were compared to control embryos for blastodisc formation, the presence of polar bodies, and cleavage pattern. Cleavage in squid is bilateral (Fig. 1a). First cleavage occurs along the line between the polar bodies and the apex of the embryo where the male pronucleus enters the egg. Second cleavage occurs perpendicular to this, and third cleavage is unequal and distinguishes the future right and left sides of the developing embryo.

Exposure to cold inhibited blastodisc cap formation in all embryos treated prior to cytoplasmic streaming; it also arrested streaming in embryos treated after second polar body formation. Twenty minutes after removal from cold exposure, precleavage stage embryos develop a blister-like swelling of clear cytoplasm surrounding the male pronucleus. Activated eggs do not form blisters of cytoplasm when removed from cold treatment, although a small crescent of cytoplasm may form over the female pronucleus after 50 minutes. Over this same period of time, the polar bodies that are present swell to more than 4 times their normal diameter of 10 μm and then slowly return to normal size. Over the next 20 min the blister of cytoplasm around the male pronucleus relaxes into a small but growing blastodisc cap that resembles a normal cap in most (95%) cases. Abnormal cap formation was observed in about 5% of the embryos examined and included displacement of the blastodisc to one side of the animal pole or splitting of the cap at the apex into two regions. Normal cleavage did not occur in these cases. In contrast to control squid embryos, which form two polar bodies, *in vitro* fertilized embryos treated during polar body formation possessed one (59/73, 37%) or two (3/73, 4%) and more frequently no (43/73 or 59%) polar bodies. Similar results were observed in activated eggs treated with cold during polar body formation. Fertilized embryos that failed to complete their meiotic divisions often possessed three nuclei at the apex of the blastodisc cap prior to cleavage, indicating that cold shock at this early stage induces polyploidy. These embryos seldom underwent normal cleavage. Interestingly, in contrast to the 2%–10% of control-activated eggs that underwent a cleavage event, 60% (79/132) of activated eggs treated with cold during their meiotic divisions possessed cleavage furrows. Embryos treated with cold from 3 to 4 h, the time when control embryos undergo first cleavage, possessed two polar bodies (as did all other embryos treated at later times), formed normal blastodisc caps, and cleaved normally. In contrast, even though first cleavage begins at 3.5 h, embryos treated from 4 to 5 h of development and returned

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**Figure 1.** Cold exposure inhibits specific cleavage furrows in the squid embryo. Individual furrows are numbered in order of their appearance. (a) Control third cleavage stage embryo, animal pole view. Note that third cleavage is unequal and that the polar bodies (pb) lie adjacent to the first cleavage furrow, which marks the midline body axis. (b) Embryo treated with cold exposure from 4 to 5 h after fertilization. The first cleavage furrow is missing, and third cleavage is equalized. (c) Embryo treated with cold exposure from 5 to 6 h after fertilization. The second cleavage furrow is missing in this embryo, while first and third cleavage furrows are present and normal. Images magnified 650X.
to room temperature failed to retain their first cleavage furrow (Fig. 1b) in 90% of the cases examined (36/40). In these embryos, because the polar bodies mark the region through which first cleavage will form, it is possible to determine that second cleavage occurred normally, while third cleavage—which is normally unequal—was equalized to mirror the cells in the future dorsal region of the embryo. Most embryos treated between 5 and 6 h of development retained a reduced first cleavage furrow at the center of the blastodisc and formed normal second and third cleavage furrows (76/89 or 85%), while other embryos from this group developed without a second cleavage furrow (Fig. 1c). This pattern was also observed in embryos treated between 6 and 7 h. Surprisingly, most of the embryos from all treatment groups continue to develop and at 48 h appeared fairly normal, although they often possessed clumps of large cells, uneven blastoderm yolk boards, and regions where cell layers appeared thicker than controls.

These results suggest that the ordered movement of cytoplasm, which forms the blastodisc in the squid, is disturbed by cold treatment. Cold exposure also induced polyploidy and perturbed cleavage furrows. The failure to retain or create specific cleavage furrows may be due to the direct action of cold on the microfilaments responsible for furrow formation, cell membranes, or specific factors that regulate mitosis. However, the formation of the blister-like swellings of cytoplasm around the male pronucleus, likely initiated by the sperm centriole to form microtubule arrays, suggests that cytoplasmic movements are rapidly resuming and may disturb the previously formed or forming microfilaments responsible for cleavage. That the polar bodies, which are little more than unwanted chromosomes and microtubules, swell rapidly during this same period further suggests that microtubules may be partially responsible for these events, although this does not rule out the possibility that cold exposure results in destabilization of membranes in these cells. Microtubules originating from the sperm pronucleus are crucial for the reorganization of cytoplasm after fertilization in frog eggs (6). The result that cold exposure can equalize third cleavage in squid embryos is nearly identical to what was reported when squid embryos were treated with the microfilament inhibitor cytochalasin B, although first cleavage furrows were still present in some of those embryos (7). To address the importance of microtubules and microfilaments alone and in concert to blastodisc formation and cleavage in the squid, it will be necessary to selectively challenge each element with specific inhibitors and characterize their appearance over time with immunohistochemistry.

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**Cytoplasmic Proteins on the Surface of Discharged Microsporidian Sporoplasms**

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The spore cell of an intracellular microsporidian parasite is a missile which, when activated, explosively discharges an invasion tube. The spore contents (sporoplasm) pass through the invasion tube and are introduced into a target cell. Evidence reported earlier indicates that the membrane surrounding the newly discharged sporoplasm cell is derived in part from the polaroplast organelle of the spore (1). An early accepted model for microsporidian sporoplasm discharge held that the membrane everts with the cytoplasmic face shifting outward during extrusion (2). If this is what happens, it would seem that cytoplasmic proteins might remain attached to this membrane and end up on the surface of the discharged sporoplasm. Evidence presented here indicates that cytoplasmic tubulin and dynactin proteins are on the surface of discharged microsporidian sporoplasms, thus supporting the idea of membrane eversion during spore extrusion.

In this study, proteins were identified from the sporoplasms discharged from spores of the microsporidian, *Spraguea lophii*. The protocol for isolating the sporoplasms was reported earlier (3). Sporoplasms examined immunocytochemically for surface tubulin (using IgG monoclonal or polyclonal primary antibody, with fluorescein-coupled secondary antibody) revealed an even, but sometimes patchy labeling (Fig. 1A). Similar results were found when sporoplasms were tested with fluorescein-labeled colchicine. In a follow-up experiment, sporoplasms were incubated in tubulin assembly medium with fluorescein-coupled tubulin. The results showed a preferential bordering of the labeled tubulin around the sporoplasms (Fig. 1B).

The *S. lophii* sporoplasms were also tested for surface dynactin proteins by using antibodies for p150glued, dynein intermediate chains, and dynein heavy chains. The site of binding was visualized with colloidal gold or fluorescein-coupled secondary antibody. The results showed some uneven labeling for p150glued (Fig. 1C) and dynein light chains, but no dynein heavy-chain labeling was apparent. Western blot analyses revealed substantial levels of p150glued and dynein light-chain proteins. The positioning of these proteins onto the sporoplasm surface suggests that the sporoplasm membrane at first faces the cytoplasm within the spore, but shifts to the outside during spore discharge.

Surface dynactin is an important component in the movement of membranous structures within cells. Recall, moreover, that *S.*
lophii parasitizes the central nervous system of different species of angler fish of the genus Lophius, the infections being particularly evident in the cranial ganglia, dorsal root ganglia, and the supramedullary neurons. Surface dynactin, therefore, is probably involved in positioning the microsporidian parasites in neuronal cell bodies within the central nervous system of their piscine hosts (4).

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### Figure 1

Spraguea lophii sporoplasms with tubulin labeling. (A) Anti-tubulin fluorescence confined to sporoplasm surface. (B) Time-interval recordings of fluorescein-coupled tubulin bordering spherical sporoplasms. (C) Anti-dynactin p150glued label with patchy positioning on sporoplasms. All scale bars represent 5 μm.

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### Adhesion of a Viral Envelope Protein to a Non-Self-Binding Domain of the Aggregation Factor in the Marine Sponge *Microciona prolifera*

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Studies of the *Microciona* aggregation factor (MAF) have thus far been focused upon its self-binding characteristics. This study demonstrates for the first time an MAF non-self-binding domain. The purpose of this study has been two-fold: (a) to purify the binding motif as a potential pharmacoactive microbicide; and (b) to characterize non-self adhesins as participants in the cross-species binding of microbes to sponge cells. Cell-cell aggregation in *Microciona prolifera* is mediated by its aggregation factor, a species-specific compound exhibiting a unique sunburst structure at high magnifications, with a molecular weight of $2 \times 10^7$ Da (1, 2). There is a bracelet-like protein core composed of multiple beads, each attached to a protein-carbohydrate arm. Multiple anionic glycans on the arms polymerize to form a viscous gel in the presence of calcium. A sulfate disaccharide and a pyruvate trisaccharide mediate self-binding to adjacent arms and to cell membranes (3, 4). A hyaluronic acid (HA)-like compound stabilizes the core-arm connections (5). Binding inhibition studies of MAF

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fractions were carried out using as a binding model recombinant (r) gp120 human immunodeficiency virus (HIV-1) envelope protein and lymphocyte rCD4 protein receptor.

MAF was prepared from cuttings of sponge branches and processed as described elsewhere (6). The clear gel derived from cesium chloride gradient ultracentrifugation was dialyzed repeatedly against 200× volume de-ionized water and then lyophilized (MAF-l). The result was a truncated molecule, as shown by electron microscopy—one having morphologically intact bracelets, but lacking arms (7). Low molecular weight anionic glycans were prepared from MAF-l by ethanol precipitation (8) followed by recovery and lyophilization of the ethanolic supernatant (MAF-ds).

**Figure 1.** Summary of the antiviral property of MAF in CEM lymphoblastoid cells infected with human immunodeficiency virus (HIV-1) (a, b). Sensorgrams indicate MAF inhibition of rgp120-rCD4 binding by MAF (c, d), whereas synthetic sulfated and pyruvylated constructs show no inhibition (e, f). (a, b) Results of formazan XTT assays for viral inhibitory properties of MAF derivatives. Round black dots and diamonds depict infected treated culture and uninfected treated culture respectively. MAF gave >50% protection at a dose as low as 0.1 μg/ml (EC50), while demonstrating little or no toxic effects. Lower dotted line demonstrates viral pathogenic effect in infected untreated culture. Straight dashes are 50% and 100% reference lines. (c–f) Sensorgrams which schematize the relative binding affinities of self and non-self MAF binding epitopes. The small letters that accompany each set of curves represent the following: a = rgp120 – rCD4 binding, b = rgp120 + putative inhibitor – rCD4 interaction, c = putative inhibitor – rCD4 reaction. The constants for these reactions are given by the terms kₐ and kₕ, where kₐ refers to the association constant and kₕ describes the dissociation constant. The values for rgp120 at the stated concentration were 1.2 × 10⁴ and 3.3 × 10⁻⁴ respectively and indicated a strong binding affinity between rgp120 and rCD4 (a curves). The reactions did not differ appreciably when gp120 was in mixture with the sulfate or pyruvylated compounds (b curves). However, both MAF₁ and MAF ds were reactive as inhibitory compounds. MAF₁ also possessed a strong affinity for rCD4, as indicated by the c curve shown on the left middle drawing.
Compositional analyses of MAFds demonstrated a high sulfate and carbohydrate content; spectroscopic analysis showed a major peak that had a mass/charge ratio of 3 kDa and represented about 60% of the solids in the dried sample. Neither fraction was active in aggregation assays at levels above 20 μg/ml, although freshly derived MAF was active at a level of 0.5 μg/ml.

Binding of the HIV-1 envelope protein gp120 to target lymphocytes via CD4 peptide is necessary for syncytium formation and viral entry and multiplication in target cells (9). Inhibition of gp120-CD4 binding by MAF fractions was evaluated using surface plasmon resonance as the detection principle for molecular interaction analysis (10). Instrumentation provided by BIACORE Company (Piscataway, New Jersey) permits ligands to be immobilized on a gold sensor chip upon which a light beam is directed. A continuous flow system permits injection of binding compounds alone or in mixture with inhibitors over the ligands. Binding causes a change in the angle of the light beam, with an association phase beginning at analyte injection and a dissociation phase at the end of injection. The changes are recorded as a sensorgram. The inhibition of HIV-1 by MAF compounds was assayed in infected lymphoblastoid cells by using a colorimetric method in which a colorless compound (formazan XTT) is metabolically converted by healthy cells, but not dead cells, to an orange-colored derivative (11).

The results of MAF titrations for two MAF fractions in formazan assays indicated that amounts as low as 0.1 μg conferred protection on more than 50% of the cells (EC50) while simultaneously showing little or no toxic effects toward non-infected cells (Fig. 1a, b). Data for the binding of recombinant rgp120 to rCD4 gave a dissociation constant of 17 nM, which agrees well with the previously reported by Wu et al. (12). At concentrations of 0.75 mg/ml, both MAF derivatives inhibited binding (Fig. 1c, d). Tests for binding inhibition using synthetic constructs and polymers of the MAF self-binding epitopes sulfated disaccharide and pyruvylated trisaccharide were completely negative (Fig. 1e, f). Of the four compounds, only MAF-1 showed any binding to rCD4 at the end of the injection cycle, while MAF-ds was bound to rgp120, but not to rCD4.

In summary, a partially purified MAF derivative (MAFds) in its chemical properties to HIV-inhibitory products derived from some other marine invertebrates (13). Although it seems unlikely that Microciona would encounter the HIV virus in its natural surrounding, we propose that this model typifies cross-species binding (as demonstrated by a non-self adhesin unrelated to the known self-binding epitopes), and it may provide the sponge with a means of immobilizing symbionts or other forms that are required for nutrition or for disposal by macrophages.

Literature Cited
Eggs of the sea urchin (*Arbacia punctulata*), when suspended in isopicnic seawater and subjected, by centrifugation for several minutes, to a gravitational field a few thousand times greater than the earth’s, stratify into several layers. As shown earlier by Harvey, who used supra vital dyes (1), the layers are, from top (centripetal pole) to bottom: oil cap, a large clear zone immediately below the oil cap containing the nucleus, the mitochondrial layer, yolk granules, and the pigment granule layer.

Observing this phenomenon with a recently developed centrifuge polarizing microscope (CPM) (2, 3), we find that centrifugation introduces to the upper part of the clear zone a negatively birefringent curtain of material that drapes down from the oil cap and surrounds the isotropic nucleus (Fig. 1A, B). The negative birefringence increases with both time and speed of centrifugation. The negative sign of birefringence (larger refractive index perpendicular to the texture), fluorescence staining with brefeldin A, and electron microscopy of fixed cells (5) suggest that this negatively birefringent material is a pleated series of endoplasmic reticular membranes, stratified and oriented by the centrifugation.

When a stratified egg is fertilized, the birefringence, viewed with the CPM, disappears in a few seconds, a surprising observation (Fig. 1C–E). Furthermore, the egg concurrently starts floating up in the Percoll-seawater density gradient (Fig. 1F–H). As the egg floats up, the fertilization envelope rises and its positive birefringence increases over the next 3 to 4 min. In the next 10 or so min, the negative birefringence below the oil cap completely disappears.

In 1952, McCulloch observed negative birefringence in the upper region of the clear zone in *Arbacia* eggs examined after centrifugation but not the changes we saw after fertilization (4). He attributed the birefringence to cytoplasmic fibrils, and with the fixatives available in those days, he was unable to discern any ultrastructure in the clear zone using an electron microscope, the only exception being the annulate lamellae, which he interpreted to be “coarse fibrous components.”
gradually returns again, but with a much more complex alignment of material.

To test whether these changes reflect a rise in cytosolic Ca$^{2+}$ (released from the endoplasmic reticulum [ER], an intracellular Ca$^{2+}$ storage organelle), we observed the responses of unfertilized stratified eggs to the calcium ionophore A23187 (6). Whether in normal or Ca$^{2+}$-free seawater, the eggs indeed responded exactly as when fertilized. We surmise that elevation of cytosolic Ca$^{2+}$ in the seconds immediately following fertilization (7) is correlated with the transient breakup of the ER (8, 9), and that this breakup is manifested as the rapid loss of birefringence. As the Ca$^{2+}$ level drops again, the ER must re-assemble into large layered sheets since the negative birefringence reappears. In control experiments, inactivated eggs, that had received the identical history of centrifugation, retain their negative birefringence for more than 30 min.

Following fertilization, the egg may become less dense due to exocytosis and swelling of the cortical granules, or by uptake of water by the egg or the egg jelly. While not observed when stratified eggs were fertilized in normal, Ca$^{2+}$-containing seawater, eggs activated with the Ca$^{2+}$ ionophore suddenly fall in the density gradient after steadily rising for several minutes. Since this fall is accompanied by a sudden release of diffuse material surrounding the fertilization envelope, swelling of the egg jelly may be primarily responsible for the increased buoyancy of the egg following its activation.

Support of research by Hamamatsu Photonics KK, Olympus Optical Company, Kyoto University, and the Marine Biological Laboratory is gratefully acknowledged. We thank Drs. B. Kaminer and M. Terasaki for extensive discussions of their work relating to changes in cytosolic Ca$^{2+}$ and ER organization following fertilization.

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**Fertilization-induced Changes in the Fine Structure of Stratified Arbacia Eggs. II. Observations with Electron Microscopy**

Mario H. Burgos$^1$, Makoto Goda$^2$, and Shinya Inoué

(Marine Biological Laboratory, Woods Hole, Massachusetts)

Unfertilized *Arbacia* eggs are stratified by centrifugation; the centripetal pole is occupied by an oil cap, which crowns a large clear zone containing the nucleus (1). When such eggs are observed with the centrifuge polarizing microscope (CPM), a curtain of negatively birefringent material, draping down from the oil cap, is introduced to the upper part of the clear zone (2). When stratified eggs are fertilized or activated by the Ca$^{2+}$ ionophore A23187, this birefringence disappears within a few seconds—even before the fertilization envelope starts to elevate. Its sign, and the fluorescent staining by brefeldin A, suggest that the negative birefringence is due to a stack of membranes, stratified and aligned by centrifugation, and oriented more or less parallel to the direction of the centrifugal force.

To evaluate this proposal further, we investigated the birefringent region of the egg by electron microscopy. We used 2% glutaraldehyde in phosphate-buffered saline made up into 700-mM sucrose to prevent swelling of the *Arbacia* egg. Eggs placed in fixative without sucrose swelled up to about eight times the volume of the unfixed egg, lost their microvilli, and (reversibly) lost their negative birefringence.

Thin sections of stratified non-activated eggs, fixed with sucrose-glutaraldehyde, retained their negative birefringence and revealed that the birefringent region is occupied by stacks of smooth and rough endoplasmic reticulum (ER; Fig. 1A). The ER surrounded the nucleus and was aligned more or less parallel to the axis of centrifugation. A small number of Golgi membrane stacks were found amidst the ER, but with random orientation. At the lower region of the ER, we found stacks of annulate lamellae (3, 4). These are most likely the refractile rod- and plate-like structures that are seen in centrifuged eggs by light microscopy, especially clearly in DIC. They tended, at first, to lie parallel to the axis of centrifugation, but changed their orientation as time elapsed after the centrifuge was stopped.

In centrifugally stratified eggs fixed about 5 min after fertilization—well after the negative birefringence had disappeared, but before it re-appeared—the distribution of the Golgi and annulate-lamellar material was basically unchanged. However, the ER was no longer in large sheets oriented along the centrifugal axis; rather, the sheets had fragmented into smaller vesicles (Fig. 1B), as was anticipated from their loss of bire-

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2 Kyoto University, Japan

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**Literature Cited**

fringence. Because the birefringence of activated live eggs does return in the upper half of the clear zone after about 10 min, albeit with less ordered alignment of the birefringence axes, the EM of cells fixed at that stage would be expected to again show stacks of large ER membrane sheets, but with the stacks oriented along less uniform axes.

These observations suggest that the birefringence observed in live eggs with the CPM is a good indicator of membrane anisotropy, distribution, and especially their dynamic changes. In addition, centrifugally fragmented mini-cells could well prove to be a useful source for several isolated membrane components of the cells.

We thank Hamamatsu Photonics KK, Olympus Optical Company, Kyoto University, and the Marine Biological Laboratory for support of this project. We also thank Louis Kerr and Christina Stamper of the MBL Central Microscope Facility for their cooperative help with electron microscopy. M.B. was supported by an MBL Chairman of the Board Fellowship.

Literature Cited
Many of us visualize the myelinated nervous system from light microscope (LM) images. Almost all white matter space is occupied by myelinated fibers, each sheath having a thickness that relates to the caliber of its axon. Myelin sheaths are made by oligodendrocytes during nervous system development. The enormous space occupied by myelinated fibers leaves oligodendrocytes so far apart that it is impossible to see connections between cell bodies and myelin sheaths in adult tissue sections.

A picture of how myelin sheaths form comes from reconstructions of morphological images. Oligodendrocyte precursors migrate to each of the regions where axons are developing. When they arrive, they replicate and send out processes, which select, ensheathe, and myelinate axons that have reached a certain size. Each process must successfully compete for the axon segment that it myelinates and then produce and maintain a sheath of identical dimensions to its neighbors. This morphological picture represents a multitude of complex process that requires coordinated expression of many gene products. At present only a very small number of these genes are known. The immediate goal of our research program is to identify new genes involved in myelination and determine their contributions.

As a first step we developed a method to identify a population of mRNAs that are important for myelination in rat brain. This mRNA population is selectively translated near sites where myelin sheaths assemble. Myelin basic protein, a dominant myelin protein, is selectively synthesized in these sites, for unlike other myelin proteins, MBP enters myelin within minutes of its synthesis. For MBP to enter myelin so rapidly, not only MBP mRNA, but also all other components needed for its translation, must be transported from the oligodendrocyte soma to each myelin sheath assembly site. We reasoned that the capacity to synthesize proteins at sites distant from the oligodendrocyte soma would not be limited to a single protein. Furthermore, as we identified other proteins synthesized near to where myelin basic protein was incorporated into myelin, we would broaden our understanding of how myelin sheaths are assembled.

David Colman and his collaborators (1, 2) provided a starting point for our studies. They showed that MBP mRNA behaves differently from mRNAs for other myelin proteins when brain samples are subjected to subcellular fractionation. MBP mRNA purifies in myelin vesicles whereas mRNAs for other known myelin-related proteins do not. We used rat brain myelin mRNA as starting material and suppression subtractive hybridization to isolate cDNAs that represent mRNAs which colocalize with MBP mRNA in myelin sheath assembly sites. In the initial study (3), we compared two different homogenization media, one isoosmotic (0.32 M sucrose) and one hypertonic (0.85 M sucrose). Although we obtained more myelin RNA from samples homogenized in hypertonic sucrose, we worried that this RNA had higher levels of contaminating RNA than samples obtained from tissue homogenized in isoosmotic sucrose. We thus conducted most (2 of 3) suppression subtractive hybridization studies with myelin RNA prepared from isoosmotic homogenates (4). However, when we counted the novel mRNAs obtained in screens with samples prepared under each condition, we found a far more diverse population was obtained when samples homogenized in 0.85 M sucrose were used (4).

Some of the mRNAs with particular relevance to myelin sheath biogenesis, SH3p13 or endophilin 3 (5) and dynein light intermediate chain (6) were obtained from the screen with the hypertonic sample (4). During the summer of 2000, we analyzed 90 (25 were sequenced) subtraction products prepared from samples homogenized in 0.32 M sucrose and 90 subtraction products (46 were sequenced) prepared from samples homogenized in 0.85 M sucrose. We used colony hybridization and hybridization of inserts prepared from mini-prep samples to identify cDNAs derived from MBP and MOBP (myelin-associated oligodendrocytic basic protein) mRNAs. These cDNAs were known to be located in oligodendrocyte processes (7), since these were highly enriched in myelin (3; Fig. 1). The remaining cDNAs were sequenced in the Bay Paul Center sequencing facility at the Marine Biological Laboratory in Woods Hole, Massachusetts. Confirming results from our recent study (4), we found that far more (30 versus 13) novel cDNAs were obtained from myelin prepared in 0.85 M sucrose. Most of these cDNAs relate to known mRNAs (Table I). A significant portion of the mRNAs generate proteins involved in regulating protein synthesis, namely eukaryotic translation elongation factors alpha and delta and ribosomal proteins L7a and L21. A few, kinesin light chain, rab7 and evectin, increase the number of proteins with recognized functions in membrane trafficking and biogenesis. We have analyzed four cDNAs from the 0.32 M sucrose subtraction product and six cDNAs from the 0.85 M sucrose subtraction product by northern blot comparisons (starting material RNA versus myelin RNA). All of them have mRNAs that are highly enriched in myelin. Among the known mRNAs analyzed so far were ferritin light chain, eukaryotic elongation factor alpha-1 and kinesin light chain.

Future studies aim to locate the mRNAs and proteins in myelinating tissue. In addition, we will use this approach to identify mRNAs located in myelin sheath assembly sites in spiny dogfish.


Optimization of Homogenization Conditions Used to Isolate mRNAs in Processes of Myelinating Oligodendrocytes

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3 Bay Paul Center for Molecular Biology and Evolution, Marine Biological Laboratory, Woods Hole, MA.
The results of this study will, in particular, help us to select appropriate conditions to homogenize dogfish brain, which exists in an environment of far higher tonicity (approximately 1 M) than mammalian brain.

This work was funded by the National Multiple Sclerosis Society grant RG2944 (RMG) and the G. Unger Vetlesen Foundation (HGM). Funds for John Engler were from the Marine Models in Biological Research Program (NSF grant, DBI-9912287). We thank the following organizations and individuals for providing supplies, facilities, and support in this project: the Marine Biological Laboratory; the Marine Science Center, Inc.; the New Bedford Biological Station; the N东西部海洋实验室; the Leif P. Kruse Laboratory for marine biological research; the New England Aquarium and its Research Laboratory; and the Alan Alda Center for Communicating Science.

Table 1

<table>
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* GenBank accession number.
† Size refers to the nucleotide sequence in the known that matches the cDNA sequence we obtained. The size of the known is in parentheses.
** Highly related to known mRNAs—there are differences evident in comparison of aligned sequences.
would like to thank Dr. Mitchell Sogin for the generous use of the Bay Paul sequencing facility.

Literature Cited

The Ipswich River Basin, which is located in northern Massachusetts and drains into the Plum Island Sound Estuary, covers a 400-km² area composed of forest, wetlands, open and agricultural land, and a gradient of low- to high-density residential and commercial land (1). Over the last century, population growth and land-use changes in the basin have altered the land cover of the watershed. The United States Geological Survey (USGS) has recently modeled the hydrology of the Ipswich River Basin using a precipitation-runoff model called Hydrological Simulation Program-Fortran (HSPF) (2). Their intent was to develop a better understanding of the effects of water withdrawal on the water budget of the river basin (1). In addition to the hydrological data that has been collected by the USGS, we have monitored nutrient loading to build a better picture of the effects of land-use change on nutrient biogeochemistry in the Ipswich river basin. To further investigate nutrient processing in the watershed, the USGS HSPF model (1) was modified to include simulation of nutrient processing on land and in the Ipswich River and tributaries. Considering the projections that have been made for urban development in the Ipswich watershed, the ability to accurately model the resulting changes in nutrient processing may be an important tool in understanding the health of the Ipswich ecosystem. It could also become an important aid in planning future development that minimizes harmful effects to the watershed.

HSPF, in addition to simulating hydrology, is capable of simulating nutrient processing, sediment transport, pH and gasses, phytoplankton, and algae dynamics in a watershed. Nitrate-processing components for stream reaches and land areas were added to the HSPF model. Initial values required by the model were obtained from a database (3) which contains parameter values used in similar HSPF projects in the northeastern United States. Nitrate processing and output from different land types was further calibrated using an empirical relationship between fractional cover of agricultural and forested land in small catchments versus nitrate concentration in the streams into which they drain (Fig. 1A, B). In this calibration, the only nitrate input was atmospheric deposition; so the differences in nitrate output between the two land types (Fig. 1B) represent the different values chosen for constants in equations governing the simulation of nutrients in the two land-use types. A more rigorous calibration of the model, which is in progress, will include comparing simulated data on nitrate concentration in the Ipswich River with data we are collecting. All simulations run on the model were driven by meteorological input for the years 1989 to 1993, but future work on the model will include adding more recent meteorological data. The base simulation was run using 1991 land-use data for the watershed; other land-use change scenarios were run by modifying the areas of different land types in certain parts of the river basin.

Calibration of the model resulted in simulated nitrate output from forest and from open plus agricultural land (Fig. 1B) that coincided with the empirical relationship for fractional cover versus nitrate concentration (Fig. 1A). The base concentration of nitrate in first-order streams draining only forested lands was approximately 10 µM, whereas the value for streams fed by agricultural and open pasture land was closer to 70 µM (Fig. 1B). Modeled nitrate transects along the main stem of the Ipswich River show a strong trend of decreasing concentration near the head of the river, followed by a slowly decreasing concentration toward the Ipswich dam (Fig. 1C). This same general trend is seen in data collected for the same month, although in a different year (Fig. 1C). Stream-flow data along the Ipswich River reveal the opposite trend: a quick increase in flow near the head of the river, followed by a slower increase moving towards the mouth of the Ipswich River (Fig. 1C). Seasonally, nitrate concentrations at the mouth of the Ipswich River reach a peak during winter and spring (Fig. 1D). Similarly, river flow at the mouth has its highest peak in the spring and another, smaller peak in the winter (Fig. 1D). Data we have collected show similar correlation between peak discharge and peak nitrate concentrations (4).

The opposite trends in stream flow and nitrate concentration along transects from the head to the mouth of the Ipswich River suggest that the decreasing nitrate concentration may be due, at least partially, to a dilution effect. The other factors contributing to diminishing nitrate concentration in the river are in-stream processes, such as denitrification and uptake by plants and algae, that can be examined using the model. One purpose of continuing to examine nutrient processing with this model is to help determine what processes are the most important contributors to the trends that have been observed and modeled.

The HSPF model can be used to examine different scenarios for land use by modifying the areas of different land types in the basin. A 12-km² residential development was modeled at different locations in the watershed. The results from those scenarios indicated that nitrate concentration would increase at the mouth of the river, and that the increase would be greater the closer the development is to the mouth of the river. The model predicts that urbanization in the watershed will have a smaller effect if it occurs farther upstream and on tributaries, as opposed to farther downstream and on the main stem of the river. One simulation of two different scenarios showed that a 12-km² residential area built in the lower watershed on the main stem would increase nitrate concentrations at the mouth of the river by approximately 5 µM, but an identical development on a tributary feeding into the main stem at the same location would produce roughly baseline conditions at the mouth. The model can be used to look more closely at the sources and sinks of nitrate in the river basin to better characterize the processing of nitrogen and other nutrients in the watershed.
This research was funded through the NSF-EPA Water and Watersheds program DEB-9726862.

**Literature Cited**


**Solute Dynamics in Storm Flow of the Ipswich River Basin: Effects of Land Use**

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The Ipswich River in northeastern Massachusetts has supplied surrounding suburban communities with water since the 1800s. With current projections of increased urbanization in the watershed (1), solute fluxes from developed areas may have an impact on the ecology of the Ipswich River. Solute fluxes from storm flow are particularly important since storms commonly flush solutes from storage reservoirs, thereby increasing the mass transfer of solutes to the aquatic system (2). The objectives of this study were to observe solute dynamics in storm flow in three first-order catchments of the Ipswich River basin to infer how increased development will affect the aquatic system.

The three catchments were selected to represent the end-members of different land-use areas commonly found in the Ipswich River basin. The catchments represent predominately urban (URB), agricultural (AG) and forested (FOR) areas. The baseline discharges were 100, 0.4 and 10 l/s at the URB, AG and FOR sites, respectively. Rain volume at each site was measured using manual rain gauges, and samples for chemical analyses were collected.
Baseline samples of stream water were collected before and after a storm that occurred 15–16 June, 2000. Each hour during the storm, filtered and unfiltered samples of stream water were collected and stage measurements were taken. Discharge was estimated from stage measurements. Stream water and rain samples were filtered immediately with glass-fiber filters, stored on ice in the field, and refrigerated at the laboratory until analysis. All filtered samples were analyzed for NH₄ colorimetrically, for Cl, NO₃, and SO₄ using ion chromatography, and for Na, K, Ca, and Mg by atomic absorption. Unfiltered samples were analyzed for acid neutralizing capacity (ANC) and pH.

Total rainfall at the sites ranged from 19 to 46 mm. Maximum stage observed was 25, 12, and 3 cm above base flow at the URB, AG and FOR sites, respectively; maximum discharges were 400, 140, and 70 l/s. Solute concentrations in rain were similar among all sites and much lower than those found in stream water. Base flow values for all solutes were lower at the FOR site than at the other, more developed sites. Sodium and Cl concentrations were high at the URB site, probably because of salting roadways in winter months. Calcium concentrations were high in the AG site, perhaps due to the addition of lime to agricultural fields. During the storm, solute concentrations in stream water of the FOR site were relatively invariant compared to the URB and AG sites (Fig. 1a–c). Concentrations of NH₄ and NO₃ increased at the beginning of the storm, and trends were similar at the AG and URB sites (Fig. 1a). Concentrations of other solutes at the URB and AG sites decreased with the onset of the storm (Figs. 1b, c). After the storm, solute concentrations at the AG site increased rapidly toward base flow values (Figs. 1b, c). Stream water discharge from the URB site was the highest of the three catchments, as was the net flux of solutes measured (Fig. 1d).

The variations in solute concentrations observed are primarily due to site-specific differences in the relative proportions of groundwater and overland flow inputs to the stream. The proportion of these inputs is commonly regulated by the type and amount of ground cover in a particular catchment. The lack of forest cover in agricultural areas and impermeable surfaces in urban settings increase overland flow inputs to streams during storms (3). In forested catchments, runoff is typically smaller than in more developed catchments because soil and vegetation allow much of the precipitation to percolate slowly to the groundwater table. In contrast, soils in predominately agricultural catchments can become quickly saturated during storms, causing larger inputs of water to enter a stream in the form of overland flow and diluting solute concentrations. As a storm subsides, solute concentrations in stream water will typically return to base flow levels as the ratio of groundwater inputs to overland flow increases. Urban settings characteristically have large amounts of impervious ground cover preventing rain from percolating to groundwater reservoirs, thereby increasing the proportion of overland flow (urban runoff) to the stream. Hence, the large decreases in solute concentrations observed at the AG and URB sites during a storm are probably due to a larger overland flow component in these catchment streams. In contrast, the increases of NH₄ and NO₃ concentrations at these sites must be due to strong sources of nitrogen in overland flow and groundwater at the beginning of a storm that may be linked to the application of fertilizers in developed settings.

Our results show that there are marked differences in the solute dynamics of storm flow among streams in areas characterized by different land uses. Because anthropogenic inputs of nitrogen are associated with the eutrophication of receiving waters, further study is required to determine the impact of increased NH₄ and NO₃ export from urban and agricultural catchments on the aquatic ecology of the Ipswich River.

**Literature Cited**

 Nitrogen loading from land is a principal cause of eutrophication of shallow estuaries (1, 2, 3). In regions such as Cape Cod, Massachusetts, which are underlain by unconsolidated sands, the major mechanism that transports nitrogen to estuaries is groundwater flow, and the major nitrogen source (primarily in the form of nitrate, NO$_3^-$) is often wastewater from septic systems (1, 2, 3). Wastewater nitrate concentrations decrease during travel in groundwater due to dilution with clean groundwater and to loss by denitrification (4). The loss of nitrogen during flow between a septic tank and receiving estuary can be calculated by determining the reduction in concentration of dissolved inorganic nitrogen relative to the change in concentration of a passive tracer that accounts for dilution.

We investigated losses of nitrate for a domestic septic system in the watershed of Quashnet River, Cape Cod. Effluent from septic systems moves downgradient within plumes containing high concentrations of nitrate. In addition, the study area has plumes derived from fertilized turf or fields. To sort out the different plumes, we measured boron (B, a passive tracer derived from laundry detergents and associated with wastewater sources [5, 6, 7]) and potassium (K, associated with both wastewater and fertilizer sources [8, 9]) in the samples of groundwater.

To calculate loss of nitrate along the plumes, we collected samples from nine wells downgradient from the septic system. Each well was furnished with 14 ports that allowed us to sample groundwater at intervals of 1–2 m. We collected 300 ml of water from 129 ports during June and July 2000 and measured concentrations of nitrate, B, and K from the middle plume do, however, fit on the lower portion of the curves for the upper plume (Fig. 2, A and B). These circumstances lead us to think that the middle plume was probably the leading edge of a plume from a septic system located farther upgradient from our septic system. We therefore used data for the upper and middle plumes in our examination of the fate of septic system nitrogen in this watershed.

Concentrations of nitrate and B diminished as water parcels aged (age, Fig. 2, C and D, calculated from Vogel equations [10] that predict time since recharge as a function of depth in aquifer). To allow for dilution, we normalized the data by expressing concentrations as NO$_3$/B (Fig. 2, E). We estimated the NO$_3$/B values we used came from a Cape Cod site near our study area, and the data dated from 1992, only a 7–8 year difference from our date of collection. We presume that differences in B were therefore a reasonable proxy for those in our study system. We calculated losses of NO$_3$ as the difference between the age 0 nitrate concentration, allowing for dilution, and the measured nitrate concentration.

Losses of nitrate in excess of dilution were quite rapid, with rates reaching 50% loss at 0.2 years (Fig. 2, F). The loss rates diminished with time, which suggests that, if these data are representative of losses elsewhere, N losses by denitrification and retention take place primarily near the septic system source. Extrapolating the curve of Figure 2 (F), we find that near-complete losses may be reached at 4.8 years, which is equivalent to 480–730 m from the septic system, assuming a travel rate of 100–150 m per year (11).

As a minimum estimate of loss, we also calculated loss relative to our highest measured NO$_3$/B ratio (Fig. 2, E, lower dashed line). If our initial NO$_3$/B ratio were closer to this measured value, our loss NO$_3$ and age continues to hold beyond our oldest sample. This would not be the case if the availability of labile organic carbon were to limit NO$_3$ loss before 100% loss is achieved.

1 Lafayette College, Easton, PA 18042.
Figure 1. Vertical cross section from the soil surface, water table, and aquifer through our field of multiple sampling wells (elevation relative to mean low water [MLW]). The numbers are concentrations of NO₃ (µM) for water samples collected from each of the 14 ports in each of the 9 wells. Although the wells were not all in one plane, for simplicity they are shown as if they were. Contour lines are drawn to indicate NO₃ concentrations of 32, 128, 512, and 2048 µM. Position of salty water determined from salinity of water samples.

Figure 2. A: NO₃ concentration versus B concentration for samples collected from upper (○), middle (■), and lower (□) plumes. B: NO₃ concentration versus K concentration for all three plumes. C: NO₃ concentration versus age for upper and middle plumes. D: B concentration versus age for upper and middle plumes. E: NO₃ to B ratio versus age for upper and middle plumes. F: Percent loss of NO₃ versus age for upper and middle plumes.
If coastal zone managers wish to regulate septic nitrogen loads, they could concentrate on management of septic systems that lie within 480–730 m of the shore, since these appeared to be the major contributors of nitrate to receiving estuaries. Septic sources farther upgradient probably contribute less significantly.

This research was supported by an internship from the Woods Hole Marine Science Consortium to Elizabeth Westgate, an MIT Sea Grant (#65591) awarded to Ivan Valiela and Harold Hemond, and National Estuarine Research Reserve Fellowships to Kevin Kroeger and Wendy Pabich.

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**Effects of Nitrogen Load and Irradiance on Photosynthetic Pigment Concentrations in *Cladophora vagabunda* and *Gracilaria tikvahiae* in Estuaries of Waquoit Bay**

Michelle Denault¹, Erica Steive, and Ivan Valiela

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Two major controls of activity and standing crop in macroalgae are nitrogen supply and irradiance (1). Increased nitrogen loads increase production (2) and biomass of macroalgae such as *Cladophora vagabunda* and *Gracilaria tikvahiae* (E. Stieve, unp. data). Lower light availability lowers growth rates of macroalgae, although this effect varies among species (3). Because of exponential attenuation and self shading within algal mats, the irradiance available for benthic algae depends on water depth (1). Supply as well as storage of nitrogen and photons affect the concentration of photosynthetic components in macroalgae (1, 4).

Photosynthetic pigments such as phycoerythrin also act as nitrogen pools, and macroalgae acclimate to different irradiance regimes by changing pigment concentrations (5).

To examine the effects of different nutrient supplies on photosynthetic pigment concentrations in a green and a red alga, we collected samples of *Cladophora vagabunda* (L.) van den Hoek and *Gracilaria tikvahiae* McLachlan from five estuaries within Waquoit Bay (Childs River, Eel River, Quashnet River, Sage Lot Pond, and Timms Pond) that are exposed to different nitrogen loads (6). To study the effect of irradiance on pigment concentration we collected samples at a range of depths (80 to 210 cm). Irradiance at each sampling depth was measured using a spherical underwater sensor attached to a Li-Cor DataLogger LI-1000. Samples were collected during one day in early June.

At each site, six samples of benthic macroalgal material were collected and sorted to isolate fronds of *C. vagabunda* and *G. tikvahiae*. The samples were sorted by species. Chlorophyll *a*, *b*, and carotenoids were extracted as described by Figueroa *et al.* (7); phycobiliproteins were extracted as described by Beer and Eshel (8). Pigments were extracted within 36 h of collecting and were kept at 5°C until extraction to avoid pigment degradation. Concentrations of pigments were determined by use of a Perkin Elmer UV/VIS spectrophotometer (8, 9, 10). Chlorophyll *a* and carotenoid concentrations were measured in both *C. vagabunda* and *G. tikvahiae*. Chlorophyll *b* concentrations were measured in *C. vagabunda*. Phycoerythrin concentrations were measured in *G. tikvahiae*. To further ascertain the internal storage of nitrogen and carbon under different nitrogen and irradiance regimes, we dried macroalgal samples and measured percent nitrogen and percent carbon in a Perkin Elmer elemental analyzer according to the manufacturer’s instructions.

Concentrations of chlorophyll *a* (Fig. 1A) and carotenoids (Fig 1B) in both species of macroalgae increased as nitrogen load to the estuaries increased. There was no consistent difference between upstream and downstream sites within the estuaries, and there was no apparent effect of different salinities at the sites of collection (range of 10‰ to 32‰) on pigment concentrations (data not shown), so data were pooled within each estuary. Concentrations of chlorophyll *a* and carotenoids in *C. vagabunda* and *G. tikvahiae* are within the range found in other published literature (5); more importantly, the nitrogen loads increase pigment concentrations from values characteristic of nitrogen-poor waters to those of nitrogen-rich estuaries (Fig. 1A, B) (5). The response of *C. vagabunda* to nitrogen supply was more pronounced than that of *G. tikvahiae* (Fig. 1A, B).

These results suggest that nitrogen supply has important effects on pigment concentrations and nitrogen content of fronds, and that the response depends on the species. Surprisingly, concentrations of phycoerythrin in *G. tikvahiae* did not increase as nitrogen load
increased (Fig. 1E), even though this pigment is known to function as a nitrogen reserve (11). *G. tikvahiae* may not be nitrogen-limited in these estuaries, an inference that we base on both the relatively low response of chlorophyll \( a \) (Fig. 1A) and carotenoid (Fig. 1B) concentrations, and the lack of response of phycoerythrin concentrations (Fig. 1C) to nitrogen load. We also measured concentrations of chlorophyll \( b \), which showed a positive response to nitrogen load (data not shown). Curiously, concentrations of chlorophyll \( b \) responded more strongly to increased nitrogen supply than did chlorophyll \( a \) concentrations (Fig. 2A). We have no explanation for this response.

Concentrations of chlorophyll \( a \) (Fig. 1D) and carotenoids (Fig. 1E) in both species of algae decreased similarly and significantly at lower irradiance. These results suggest that the mechanism of response to irradiance is similar for both species of algae. Phycoerythrin concentrations (Fig. 1F) did not change with differences in irradiance. The percent N content of *C. vagabunda*, but not of *G. tikvahiae*, increased in parallel to nitrogen load (Fig. 2B, C). The increase in nitrogen content in *C. vagabunda* is even more striking in view of the decrease of carbon content in *C. vagabunda* as nitrogen load increased (Fig. 2B). Percent nitrogen in *G. tikvahiae* fronds did not increase significantly with nitrogen load, in agreement with the results of Figure 2C.

Increased nitrogen loads were paralleled by significant increases in photosynthetic pigments in *C. vagabunda* and *G. tikvahiae* as well as by an increase in percent N in *C. vagabunda*, but not in *G. tikvahiae*. These results suggest that the supply of nitrogen may be sufficient to support growth in *G. tikvahiae*, but that *C. vagabunda* is nitrogen-limited in those estuaries of Waquoit Bay that receive the lowest nitrogen load from land. This conclusion is verified by biomass data (E. Stieve, unpub. data) that show that the response of standing crop of *C. vagabunda* is a function of nitrogen load, and that increased (Fig. 1E), even though this pigment is known to function as a nitrogen reserve (11). *G. tikvahiae* may not be nitrogen-limited in these estuaries, an inference that we base on both the relatively low response of chlorophyll \( a \) (Fig. 1A) and carotenoid (Fig. 1B) concentrations, and the lack of response of phycoerythrin concentrations (Fig. 1C) to nitrogen load. We also measured concentrations of chlorophyll \( b \), which showed a positive response to nitrogen load (data not shown). Curiously, concentrations of chlorophyll \( b \) responded more strongly to increased nitrogen supply than did chlorophyll \( a \) concentrations (Fig. 2A). We have no explanation for this response.

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**Figure 1.** Milligrams dry weight of pigments per gram dry weight of macroalgae are shown versus nitrogen load (left column) and versus irradiance (right column). (A) Chlorophyll \( a \) (*C. vagabunda*, \( F = 32.83, P < 0.001; G. tikvahiae, F = 6.86, P < 0.05 \)), (B) carotenoid (*C. vagabunda*, \( F = 26.88, P < 0.001; G. tikvahiae, F = 6.61, P < 0.05 \)), and (C) phycoerythrin (ns) concentrations versus nitrogen load. The dotted lines show published (5) pigment concentrations ranges for macroalgae in nitrogen-poor waters (lower dotted lines) and nitrogen-rich water (upper dotted lines). (D) Chlorophyll \( a \) (*C. vagabunda*, \( F = 8.52, P < 0.01; G. tikvahiae, F = 7.50, P < 0.05 \)), (E) carotenoids (*C. vagabunda*, \( F = 6.71, P < 0.05; G. tikvahiae, F = 10.60, P < 0.01 \)) and (F) phycoerythrin (ns) concentrations versus irradiance.

**Figure 2.** (A) Chlorophyll \( b \) concentration versus chlorophyll \( a \) concentration (\( r^2 = 0.785 \)). (B) Percent carbon (*C. vagabunda*, \( F = 19.17, P < 0.01 \); *G. tikvahiae*, \( F = 3.06, \text{ns} \)) and (C) percent nitrogen (*C. vagabunda*, \( F = 34.56, P < 0.001 \); *G. tikvahiae*, \( F = 0.99, \text{ns} \)) versus nitrogen load.
standing crop of *G. tikvahiae* responds less to nitrogen supply than to seasonal changes in light availability. The increase of chlorophyll a and carotenoid concentrations in *C. vagabunda* and *G. tikvahiae* in response to high irradiance is paralleled by biomass data taken from the estuaries (E. Stieve, unpub. data). The biomass of *G. tikvahiae* was greater than that of *C. vagabunda* in low-nitrogen estuaries that also furnished high irradiance to algae.

The physiological changes in photosynthetic pigments and nitrogen concentrations created by increased loads suggest increased growth of at least the green alga *C. vagabunda*. Increased nitrogen load may also increase phytoplankton standing crop, increasing light attenuation in the water column and therefore decreasing the growth of light-limited benthic algae. On balance, the growth-stimulating effect of increased nutrients seems to more than compensate for the detrimental effect of light attenuation from the influence of phytoplankton shading at the time of sampling; and *C. vagabunda*, in particular, proliferates and causes macroalgal blooms in nitrogen enriched estuaries.

This research was supported by the Woods Hole Marine Science Consortium. Special thanks to Jennifer Hauxwell for help with algae and Jennifer Bowen for her endless patience.


### Differences in Properties of Salt Marsh Sediment Between Hayed and Reference Sites

**Adena Greenbaum (Wellesley College) and Anne Giblin**

The practice of haying salt marsh grasses began in colonial times. Early settlers began harvesting marsh grasses for fodder, and the practice has continued to the present (1). Current haying techniques remove more than 90% of aboveground plant biomass, and could have a number of effects on processes within the marsh. Salt marsh food webs are based on detritus, so it can be hypothesized that removal of plant biomass could alter food webs. Nutrient cycles, benthic algal biomass, microbial processes, and species composition could also be affected (2, 3). This study examined the effect of detritus removal on several sediment properties to assess the long-term effects of haying.

Sediment cores were taken from Plum Island Sound intertidal marsh, a long-term ecological research site located in northeastern Massachusetts. To study the effects of detritus removal, we measured several characteristics of sediment in areas where the marsh grass is hayed by commercial farmers. We compared the results to those of reference areas, which are not hayed. We sampled two areas that are hayed every other year, a practice the commercial farmers recognized produced the highest hay yield.

Duplicate cores were taken from each of two hayed and reference sites. All cores were taken from high marsh areas that were heavily dominated by *Spartina patens*. Measurements of the following sediment characteristics were taken at 2 cm intervals above 10 cm and 5 cm intervals below 10 cm, to a depth of at least 25 cm. Bulk density, a measure of soil density, was expressed as ratio between the weight and volume of sediment; percent organic matter was measured by loss of ignition; total sulfur was measured using a LECO sulfur analyzer; sedimentation rates were calculated using Pb profiles (4); and total phosphorus was measured using the technique of Krom and Berner (5). These properties were measured to examine sediment composition.

There was no significant difference in bulk density between surface sediments in the hayed and reference sites (Fig. 1). Bulk densities for both the hayed and reference areas decreased from a range of 0.37 to 0.34 g cm\(^{-2}\) at the surface to 0.22 g cm\(^{-3}\) at 12.5 cm. Below this depth, there was a slight difference in bulk density values. The hayed areas increased to a maximum of 0.34 g cm\(^{-3}\) at 23 cm. However, the bulk density of the reference sites remained around 0.23 g cm\(^{-3}\). Judging from surface values for bulk density, current haying practices apparently do not compact the sediments.

Percent organic matter was similar between the managed and natural areas to a depth of 12.5 cm. Values ranged from 31.7% to 44.5% organic matter. Deeper sediment samples of reference plots had a slightly higher percent organic matter than the hayed sites, but the difference between them was not significant. This indicated that the removal of biomass from the hayed sites does not affect organic matter content.

Haying did not appear to affect the total sulfur content in the sediment. In both hayed and reference areas, total sulfur increased from about 0.65% at the surface to a maximum of 2.2% between 17 and 22 cm, and then decreased to about 1.5% at a depth of 30 cm.

1 Ecosystems Center, Marine Biological Laboratory, Woods Hole, MA.
Values between hayed and reference sites for percent total sulfur, along with bulk density and percent organic matter deviated slightly below 12.5 cm. This sediment was deposited more than 25 years ago, and since land practices before this time are unknown, it is difficult to hypothesize reasons for the deviation.

Sedimentation rates can be estimated by using stable lead profiles to approximate dates of deposition. The lead profiles for hayed and reference areas were not significantly different. Although the cores were not deep enough to reach pre-industrial background values, we used a previous study to establish background levels (Schmitt, unpub. data). Using both sets of data, we calculated a sedimentation rate of 0.54 cm y
2
1 for both the reference and the hayed areas. The lack of differentiation in sedimentation between the hayed and natural marsh is surprising given that the haying process removes a large portion of the aboveground biomass. One possible explanation is that most of the organic matter making up the peat comes from other sources. Belowground biomass could contribute significant amounts of organic matter to detritus, especially since a large percent of biomass in a salt marsh could be below ground. Organic matter washed in with the tide could settle as detritus as well. The profile for the hayed sites was more variable than that of the reference site. Perhaps the tractor and trailer used for haying disturbs the surface sediment as it travels over the marsh.

There was a significant difference between total phosphorus measured in hayed and reference sites. The hayed areas had less total phosphorus than the reference sites from the surface until 22.5 cm deep in the sediment, where the values for the two areas converged. One explanation is that the input of phosphorus to the marshes of the area is very low, and the periodic removal of biomass from the system eventually leads to a measurable loss in phosphorus.

Of the sediment properties we examined—bulk density, percent organic matter, total sulfur, sedimentation rate, and total phosphorus—only the last was affected by harvesting the aboveground biomass every other year. Current haying practices on the marsh did not significantly alter most of the properties we measured. However, other processes in the marsh could be affected by the decrease in phosphorus, and other systems could respond differently to comparable practices.

This research was supported by the Plum Island Sound LTER and a Research Experience for Undergraduates NSF fellowship. Thanks to Linda Deegan, Hap Garritt, and Nat Weston for advice and assistance with sampling.

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Population Genetic Structure of the Goosefish, *Lophius americanus*

Hemant M. Chikarmane, Alan M. Kuzirian (Marine Biological Laboratory, Woods Hole, Massachusetts), Robbin Kozlowski\(^1\), Mark Kuzirian,\(^2\) and Tony Lee\(^3\)

*Lophius americanus* Cuvier & Valenciennes 1837 (1), the goosefish, anglerfish, or monkfish, is common in coastal waters of the northeastern United States. Its geographic range extends from the northern Gulf of St. Lawrence south to Cape Hatteras, North Carolina (2, 3). The highest fish concentrations are found along the shallower depths of the shelf from 70 to 100 m, but there is also a significant deep-water population below 190 m. Adult fish migrate seasonally in response to spawning, food availability, and optimal temperatures (\(3^\circ–9^\circ\)C) (2). The species is also dispersed through the drifting of egg rafts. Total dispersal time from embryonic development through larval and juvenile stages can extend to several months until benthic recruitment occurs. Sexual maturity is reached between 3 and 4 years of age (3).

Goosefish is the fourth largest commercial species in the U.S. fishery, and number one in demersal species landings. Goosefish landings have risen steeply through the 1980s, reaching approximately 28,800 mt ($35 million) for 1997 (4). Since the 1980s, the Canadian contribution to the fishery has declined precipitously, and now the major landings occur in the southern regions of the species range. In their autumn survey data, the Northeast Fisheries Science Center, Woods Hole, Massachusetts, has documented recent sharp declines in goosefish abundance, from 2.24 kg/tow in 1986 to 0.74 kg/tow in 1996. The New England and Mid-Atlantic Fishery Management Councils (NEFMC and MAFMC) consequently designated goosefish as overexploited and at low abundance (5). The 23rd Stock Assessment Workshop at the Northeast Fisheries Science Center concluded that it was not possible to delineate the stock structure for goosefish because of the lack of genetic, tagging, or migration studies. Nevertheless, the Councils divided the coastal population into northern and southern stocks (41°N latitude) for stock management purposes. This formula led to fishing restrictions being placed geographically, and made certain areas uneconomical to fish. Because of the lack of definitive stock data for goosefish (5), we undertook a population genetic study of goosefish in eastern waters from the Canadian border to North Carolina. We used random amplification of polymorphic DNA and PCR (RAPD-PCR) (6) to analyze the genetic structure of the sampled populations.

Eight representative sampling sites were chosen, extending from Maine (42°40' N, 68°20' W) to North Carolina (35°40', 75°00'). Fish were collected from September 1999 to June 2000. Up to 45 fish were sampled at each location. Tissue samples were collected in tissue preservation buffer (7). Genomic DNA was purified by standard phenol-chloroform procedures, and was finally dissolved in Tris-EDTA (TE) buffer (8). DNA fingerprinting was performed by RAPD-PCR (6), using 10 \(\mu\)l per reaction. Amplification products were separated by electrophoresis on 1.2% agarose gels in 0.5x TBE (8). Gels were stained with ethidium bromide and photographed under UV light. The presence or absence of amplification products was scored manually. Cluster analysis was performed with the RAPDistance package (9).

Six fish, three each from Georges Bank and New York/New Jersey sites were first screened with the seven primers shown in Table I. As expected, the number of amplification products per primer varied, ranging from 2 to 9, and very few bands were polymorphic (Table I). On the basis of the initial screening, a subset of 6–8 DNA samples from each site was analyzed, using primers 101 and 103; Figure 1 shows data for primer 103.

There appeared to be no significant differences between individuals or between populations, with either primer. Polymorphic bands were present in a minority of individuals, usually one or two. A set of eight fish collected off Martha’s Vineyard, Massachusetts, by the Marine Biological Laboratory, was examined with an additional set of primers (115, 119, 130, and 143). Again, the band distribution was very homogeneous (data not shown). Of the 22 identifiable bands produced by these primers, 21 were present at a frequency of 100%. Band 22 was present at a frequency of 58%. All the MBL samples were clustered as one group by the RAPDistance package. These results taken together imply that the fish populations are relatively homogeneous genetically across all geographic sampling sites, the level of polymorphism within populations being as low as that between populations. Fish caught at shallower (<200 m) depths could not be differentiated from those at lower (>200 m) depths, neither could those collected north or south of the 41°N line. For the primers tested, there was no amplification product (or the absence of one) that uniquely characterized a particular population. The trend in the data is clear even though only a subset of samples was analyzed with two primers. We are currently examining the entire sample set with more primers to reinforce the validity of our results.

Table I

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Number of bands</th>
<th>Polymorphic bands</th>
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<tr>
<td>101</td>
<td>GCGGCGCGGAG</td>
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<td>1</td>
</tr>
<tr>
<td>103</td>
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</tr>
<tr>
<td>108</td>
<td>GTATGGCCCT</td>
<td>7</td>
<td>1</td>
</tr>
</tbody>
</table>

The primer numbering system and sequence is from the University of British Columbia RAPD primer kits (10).

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1 Cape Cod Community College, W. Barnstable, MA.
2 University of Rhode Island, Kingston, RI.
3 Duke University, Durham, NC.
The homogeneity of the goosefish populations off the eastern coastline of the United States suggests that there is unrestricted gene flow across the region. This is very plausible considering the preferred temperature profile and migratory patterns of the adults, and the long dispersal times of the embryos, larvae, and postlarval juveniles (2, 3). These data will have serious implications for management of the goosefish fishery. The study results run counter to the current NEFMC/MAFMC policy of dividing the fishery into northern and southern stocks. Any management plan will be difficult to implement because the spawning stock biomass is unknown. More data is also needed to determine the location of the standing reproductive population, and to assemble specific temporal data on when spawning occurs over the fish’s geographic range. Integration of the published data on seasonal abundances (NMFS Spring/Autumn Bottom Trawl Surveys) with yearly temperature profiles along the coastlines might suggest some possible avenues to pursue these answers. Such data will assist in defining the natural and fishing mortality rates (F) and what the F_{threshold} should realistically be for this commercially important species.

This work was supported in part by the Monkfish Defense Fund. H.M.C. and A.M.K. are indebted to Kathy Downey of the MDF for acquainting them with the problems of the goosefish fishery, and for organizing the fishermen for sample collection. We thank the Aquatic Resources Division, MBL, for collecting some goosefish used in this study.

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