the extensive cytoskeletal system in these algae (*15*) suggests several possibilities in using *B. forbesii* for studying the functions of microtubules and microfilaments in endocytosis and the development of coated membranes. The size and abundance of coated organelles, combined with their easily inducible and predictable occurrence, make this unusual alga a model system for isolating and characterizing coated membranes.

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7. Cells were fixed with 0.5 percent glutaraldehyde and 0.2 percent osmium tetroxide in cacodylate buffer containing salts to give a final tonicity of 900 mosmol/kg, *p*H 7.4, for 10 minutes at room temperature. After rinsing and desalting, cells were stained en bloc with 0.5 percent uranyl acetate for 60 minutes on ice. Acetone dehydration was followed with infiltration and embedment in Epon-Araldite. Sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (JEOL JEM-100CX) at 80 kV. Ferritin labeling was accomplished by wounding cells in 5 ml of growth medium (*2*) containing 2.3 mg of ferritin per milliliter (Miles-Yeda, cationized, lot CF8) at room temperature. After 60 minutes the cells were washed twice with growth medium and fixed as above.
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9. Photographs of entire intact cells and of the same cells after protoplast formation were analyzed. After the photographs were video-digitized, image enhancement removed protoplasts below the upper focal plane in the photographs, permitting analysis solely of the upper half of each cell (that portion in sharp focus). Absolute values in square millimeters were obtained by incorporating original magnifications of micrographs into the program. The initial cells chosen were nearly cylindrical, so the planar area determined by the computer was multiplied by π to give the total plasma-membrane surface area in the intact cells. This value for cell 2 in Table 1 was close to the value calculated through direct geometrical analysis (by hand) of the micrograph (14.57 mm^2), verifying accuracy of the program and of our assumptions in the procedure. The protoplasts resulting from wounding are nearly spherical, so their two-dimensional areas (πr^2) were summed for each cell by the computer and multiplied by 4 to give the total surface area. This value was then doubled, since only half of each cell was in sharp focus (due to their large size) and therefore analyzed. Accuracy of the protoplast values was checked mathematically by measuring the diameter of each of the protoplasts that formed after wounding and calculating the total surface area knowing that no cell wall is present yet and that each has a smooth surface membrane (unpublished observations). These values were consistently lower than those calculated by the computer, indicating that the decreases (Table 1) are conservative.
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Adsorption to Fish Sperm of Vertically Transmitted Fish Viruses

Abstract. *More than 99 percent of a vertically transmitted fish rhabdovirus, infectious hematopoietic necrosis virus, was removed from suspension in less than 1 minute by adsorption to the surface membrane of sperm from two genera of salmonid fishes. The vertically transmitted, infectious pancreatic necrosis virus adsorbed to a lesser degree, but no adsorption occurred with a second fish rhabdovirus that is not vertically transmitted. Such adsorption may be involved in vertical transmission of these viruses.*

In a review of vertical transmission of viruses, the role of semen in viral transmission was pointed out as being largely neglected by virologists (*1*). Most studies of mammalian viruses in semen have dealt only with venereal (horizontal) rather than vertical transfer (*2*). Investigators have rarely discriminated between virus in semen unassociated with sperm (*3*) and viruses within the sperm cell (*4*), but surface adsorption of virus to sperm cells has been described only for simian virus 40 with sperm from a rabbit, an atypical host (*5*). In oviparous species with external fertilization, venereal infection by viruses in semen is less likely to occur than vertical transmission. In vertical transmission, virus closely associated with the sperm would be more efficiently transferred to the egg than unassociated virus. We now report evidence that a vertically transmitted fish rhabdovirus adsorbs rapidly to fish sperm, whereas a closely related rhabdovirus not vertically transmitted does not.

Infectious hematopoietic necrosis (IHN) is a common rhabdoviral disease of salmonid fishes found on the west coast of North America and can be transmitted both horizontally (from fish to fish) and vertically (from generation to generation). The disease has occurred in fish in other parts of the continent, apparently associated with the shipment of eggs from infected fish (*6*). Both the history of the disease and the characteristics of the virus have been reviewed (*7*). To determine whether IHN virus adsorbs to sperm and thus whether it is involved in vertical transmission, we obtained milt from several steelhead (anadromous rainbow) trout (*Salmo gairdneri*) and pooled them (6.8×10^9 sperm per milliliter); the pooled milt was centrifuged, the seminal plasma was decanted and saved, and the sperm cells were washed twice with diluent (cell culture medium without fetal bovine serum). The milt was assayed for the presence of IHN virus; no virus was found. The sperm was then divided into equal portions and centrifuged, and 1 ml of an IHN virus suspension was added to each of six sperm pellets and to each of six control tubes containing a volume of diluent equal to the volume of sperm. The mixtures were incubated at 15°C for 1 hour with continual agitation and then centrifuged, and the virus remaining in the supernatants was measured by a plaque assay (*8*). The presence of sperm reduced the amount of virus remaining in the supernatant by 99.6 percent compared to controls (Table 1).

To determine whether reduced virus counts were due to inactivation by residual seminal plasma, we sterilized by filtration the fluid recovered from the initial centrifugation of the milt and added it to a suspension of IHN virus at a ratio of 9 to 1 (by volume). Control tubes received an equal volume of diluent. After 1 hour the amount of virus remaining in the supernatants was measured. Seminal plasma did not inactivate the virus (Table 1). For all further work, the seminal fluid was not removed from the sperm.

Cavity fluid is a viscous liquid expelled by female fish along with the eggs and can contain high concentrations of IHN virus. We tested its influence on adsorption of virus to sperm by adding cavity fluid from virus-free steelhead trout to IHN virus at a ratio of 9 to 1 (by volume). Sperm was added to one group of tubes, and diluent was added to control tubes. After incubation, more than 99 percent of the virus had been removed

Table 1. Adsorption of fish viruses onto sperm (S) or erythrocytes (E) from steelhead trout (ST), rainbow trout (RT), and chinook salmon (CS). Virus amounts expressed as arithmetic mean plaque-forming units (PFU) per milliliter ± standard error of the mean. Means were calculated from six replicate values except for the rainbow trout erythrocyte experiment which had five replicates.

Additions		Virus remaining in supernatant (PFU)		Virus adsorbed (percent)*
Fluids	Cells (donor)	With S or E	Control	
		IHN virus		
	S (ST)	$1.6 \times 10^2 \pm 8.6$	$3.7 \times 10^4 \pm 3.0 \times 10^3$	99.6
Cavity fluid	S (ST)	$2.0 \times 10^2 \pm 9.0$	$4.1 \times 10^4 \pm 3.7 \times 10^3$	99.5
Seminal plasma		$5.1 \times 10^4 \pm 3.5 \times 10^3$	$5.0 \times 10^4 \pm 2.4 \times 10^3$	0
	S (RT)	$6.3 \times 10^3 \pm 9.8 \times 10^2$	$5.9 \times 10^4 \pm 3.6 \times 10^3$	89.3
	E (RT)	$1.5 \times 10^5 \pm 3.0 \times 10^4$	$1.3 \times 10^5 \pm 5.8 \times 10^3$	0
	S (CS)	$5.4 \times 10^3 \pm 4.0 \times 10^2$	$1.0 \times 10^5 \pm 8.6 \times 10^3$	94.6
		VHS virus		
	S (CS)	$6.5 \times 10^4 \pm 5.3 \times 10^3$	$5.5 \times 10^4 \pm 3.9 \times 10^3$	0
		IPN virus		
	S (RT)	$3.1 \times 10^4 \pm 4.5 \times 10^3$	$4.1 \times 10^4 \pm 4.5 \times 10^3$	24.4

*Calculated from the mean values of virus remaining in the supernatant by $(1 - A/B) \times 100$, where A is the amount adsorbed onto S or E and B is the amount in control.

by the sperm despite the presence of the cavity fluid (Table 1), indicating that cavity fluid does not interfere with virus attachment. This observation suggests that, during fertilization, sperm from virus-free males could adsorb virus originating in the female.

We added erythrocytes from nonanadromous rainbow trout (*S. gairdneri*) to IHN virus to determine whether the removal from suspension of IHN virus by sperm was a membrane phenomenon not restricted to sperm. There was no adsorption of virus by the erythrocytes, whereas an 89.3 percent reduction in virus was observed in the control containing rainbow trout sperm (Table 1).

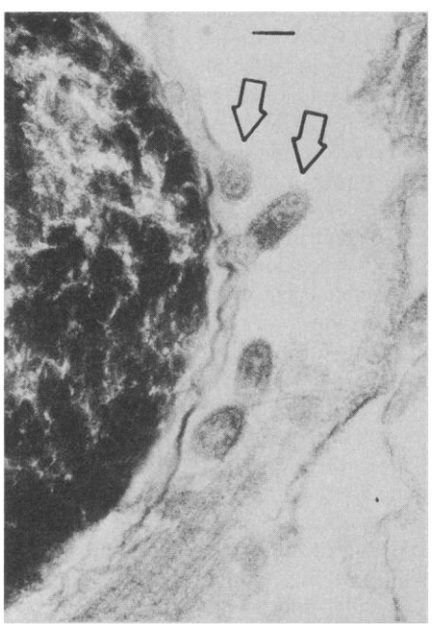

Fig. 1. Adsorption of IHN virus (arrows) to the surface of steelhead trout sperm. Equal volumes of virus (1×10^5 plaque-forming units per milliliter) and sperm (2×10^9 cells per milliliter) were combined and incubated at 15°C with gentle mixing for 1 hour. The sperm cells were removed by centrifugation and prepared for electron microscopy (*12*). Measurement of the virus before and after the presence of sperm indicated a 98.3 percent reduction. Scale bar is 100 nm.

The rhabdovirus causing viral hemorrhagic septicemia (VHS) in trout is found only in Europe but is physically and biochemically similar to IHN virus (*9*). Unlike IHN virus, VHS virus is not vertically transmitted. Therefore, we were able to study the relation of adsorption of virus to sperm and the mode of viral transmission. No adsorption of VHS virus to sperm of chinook salmon (*Oncorhynchus tshawytscha*) was observed, an indication that the adsorption process does not occur with all fish rhabdoviruses. Chinook salmon sperm were as effective as steelhead trout sperm in removing IHN virus, adsorbing 94.6 percent of the virus (Table 1). This observation suggests that adsorption of virus to sperm plays a role in vertical transmission of IHN virus and that the decrease in virus concentration after exposure to sperm cells cannot be solely due to mechanical entrapment of virus particles by sperm cells during centrifugation. Chinook salmon are natural hosts for IHN virus, and this result shows that adsorption of IHN virus to sperm occurs in other genera of the family Salmonidae.

The virus adsorption appeared to be a surface effect in that most of the virions were attached to the sperm head membrane by the flat end of the particle without penetration of the sperm cell, as revealed by electron microscopy (Fig. 1). Occasional virions were observed apparently attached on their sides. Virions attached to sperm tails were seen only rarely.

We tested a third fish virus for adsorption to sperm. Infectious pancreatic necrosis virus, an unclassified, nonenveloped, icosahedral virus with a bisegmented RNA genome (*10*), can be vertically transmitted (*11*). IPN virus adsorbed to rainbow trout sperm at a significant but lower proportion (24.4 percent) compared to IHN virus (Table 1). The occurrence of adsorption to sperm of two vertically transmitted fish viruses and the lack of adsorption of a virus not vertically transmitted supports the hypothesis that sperm plays a role in such transmission.

The kinetics of IHN virus adsorption to sperm was studied by combining equal amounts of virus and sperm suspensions in centrifuge tubes and assaying for virus remaining in the supernatant after incubation for time periods ranging from 1 minute (the shortest incubation period within which the necessary manipulations could be done) to 2 hours. The control groups received diluent instead of sperm, and viral assays on controls were performed only at time zero and at 2 hours. Virtually all IHN virus adsorbed to sperm in less than 1 minute (Fig. 2). The concentration of virus decreased

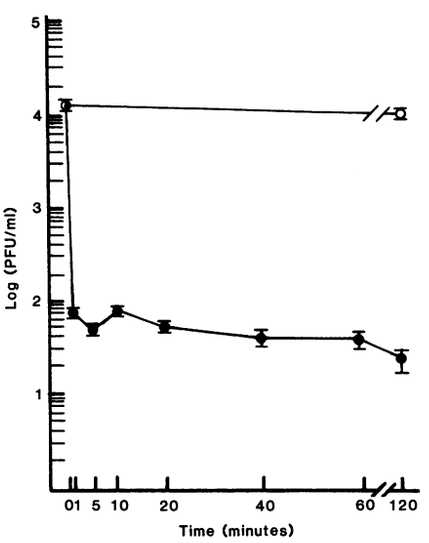

Fig. 2. Kinetics of IHN virus adsorption to steelhead trout sperm. Equal volumes of sperm and IHN virus suspensions were combined in six replicate tubes and mixed gently at 15°C for various times before sperm were removed by centrifugation from the experimental groups (●), and the virus remaining in the supernatant was assayed by plaquing (*6*). The control groups (○) received diluent instead of sperm. The virus concentration used was 6.0×10^4 plaque-forming units per milliliter and the sperm concentration was 1.4×10^{10} cells per milliliter.

slightly over the next 2 hours in both experimental and control groups. This rapid rate of adsorption was in contrast to that in the plaque assay, which required a minimum 1-hour inoculum adsorption period for maximum adsorption of virus. This difference suggests that the two systems may have different receptor sites and attachment mechanisms.

Vertical transmission of virus by attachment to sperm cells has obvious advantages for infection of any host, but particularly when the host is a species in which fertilization takes place externally and in flowing water. Without adsorption, virus particles released with gametes during spawning would be immediately diluted and flushed away by the water. Adsorption of virus to sperm could deliver the pathogen directly to the ovum, with the sperm acting as a vehicle for the entry of the virus into the egg. Because of dilution, adsorption of virus to sperm must occur very quickly—a requirement that our results indicate is met. We have also found that adsorption occurs efficiently over the range of temperatures from 1.5° to 18°C—approximately the temperature range preferred by salmonids for spawning. Our finding that IHN virus adsorbs to sperm from two genera known to be natural hosts for the virus suggests that this mechanism of transmission may occur in all host species.

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Energy Scaling in Marsupials and Eutherians

Abstract. *Marsupials have been shown to have basal metabolic rates below those of eutherian mammals. Now metabolic rates below thermoneutrality are found to be equivalent in both taxa. Two models are proposed to explain the observed metabolic patterns: in one, marsupials differ only in having reduced basal metabolic rates; in the other, the reduced marsupial basal metabolic rates combined with a reduced body temperature and elevated conductance. The metabolic costs of existence below thermoneutrality appear to be similar for both taxa. The difference in basal metabolic rate may be of energetic significance or merely a phylogenetic coincidence.*

The minimal resting metabolic rate within thermoneutrality (basal metabolism) of marsupial mammals has been shown to be 70 percent of the corresponding eutherian level at any body mass (*1, 2*). Marsupials also are considered to have lower body temperatures than eutherians (*1, 3*). These considerations led Dawson (*3*) to construct a model with the metabolism of marsupials at temperatures below thermoneutrality also being lower than eutherians (Fig. 1A). Our allometric analyses of measured metabolic rates below thermoneutrality indicate that the marsupials do not have reduced metabolic levels except within thermoneutrality and that a more accurate model is that depicted in Fig. 1B. Our analysis is consistent with the findings that the maximum rate of metabolism in marsupials and eutherians is similar in response both to cold (*4*) and to exercise (*5*) and that the daily energy expenditure in natural settings is similar in both taxa (*6*).

Dawson's model (*3*) (Fig. 1A) shows the well-documented reduction in basal metabolism of the marsupials and the lower body temperature; the latter is shown by x-intercepts: the line relating metabolism to air temperature intersects zero metabolism at a lower temperature in marsupials. The shallower slope of the marsupial line below thermoneutrality indicates a lower minimal thermal conductance than in eutherians (*7, 8*); however, at least some smaller marsupials have the same conductance as eutherians (*1*). Figure 1A also indicates that marsupial metabolism is reduced below that of eutherians at all temperatures below thermoneutrality. This would be the case if the generalized marsupial followed "Newtonian cooling" [figure 1 in (*9*)] with a body temperature below that of eutherians and a thermal conduc-

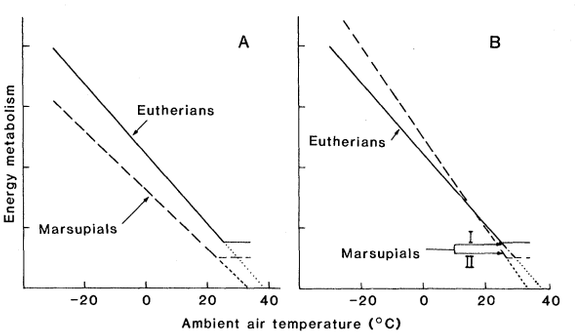

Fig. 1. Models of the relation of energy metabolism (H_m) to ambient air temperature (T_a) in marsupials and eutherians (A) from Dawson (*3*) and (B) proposed herein. The models (*7*) show a range of T_a's across which H_m is minimal and does not vary (horizontal lines). This T_a range is the zone of thermoneutrality and the H_m is often referred to as basal metabolism. At T_a's below thermoneutrality, metabolism follows the relationship $H_m = h(T_b - T_a)$ (*15*). For an animal following Newtonian cooling (*9*), the line relating H_m to T_a below thermoneutrality will project to a T_a equal to body temperature (T_b) when metabolism is zero. (B) Marsupials are shown to have a lower basal metabolism (dashed horizontal line) than eutherians in both I and II. In I, T_b and thermal conductance of marsupials are proposed to be similar to eutherians: they would then both follow the solid line below thermoneutrality. Alternatively, in II, marsupials are proposed to have a lower T_b but higher thermal conductance: marsupials would fall on the dashed line below thermoneutrality.