Methods for Nonlethal Gill Biopsy and Measurement of Na^+, K^+-ATPase Activity

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A gill biopsy, in which a small portion of gill tissue was removed from anesthetized fish, was shown to have no detrimental effect on subsequent survival, growth, and salinity tolerance of juvenile Atlantic salmon (*Salmo salar*). A method for measurement of Na^+, K^+-ATPase activity in this small amount of gill tissue is presented. These methods are useful for nonlethal monitoring of physiological smolt characteristics in salmonids and may have applications in the study of disease, toxicology, and physiological ecology of many fish species.

Une biopsie des branchies, qui consiste à prélever un petit fragment de tissu branchial chez des poissons anesthésiés, n'a pas eu d'effet nuisible sur la survie, la croissance et la tolérance à la salinité de juvéniles de saumons de l'Atlantique (*Salmo salar*). L'auteur décrit une méthode de mesure de l'activité de la Na^+, K^+-ATPase dans le petit morceau de tissu prélevé par biopsie. Cette méthode s'avère utile au contrôle non létal de caractéristiques physiologiques de smolts de salmonidés et pourrait sans doute avoir des applications dans l'étude de la pathologie, de la toxicologie et de l'écologie physiologique d'un grand nombre d'espèces de poissons.

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Na^+, K^+-ATPase is an ion-translocating enzyme present in all animal cells. It is present in extremely high concentration in salt-transporting tissues such as the vertebrate kidney, salt glands of birds, rectal glands of sharks, and chloride cells of teleosts (Karnaky 1986). In chloride cells, Na^+, K^+-ATPase creates ionic and electrical gradients that are used for salt secretion in seawater, and possibly for ion uptake in fresh water (Silva et al. 1977; Zadunaisky 1984; Pequeux et al. 1988; Avella and Bornancin 1990).

In addition to its fundamental importance to ion transport in fish, gill Na^+, K^+-ATPase activity is often used as an indicator of the parr–smolt transformation of juvenile salmonids in hatcheries and laboratories. Zaugg (1982) presented a simple and widely used method for measurement of gill Na^+, K^+-ATPase activity; however, this and other methods require relatively large amounts of gill tissue, which requires killing juvenile fish. This paper validates a method of gill biopsy and measurement of Na^+, K^+-ATPase that should be useful for nonlethal sampling of young salmonids and other fishes. In addition to monitoring physiological changes in anadromous salmonids, gill tissue is important in monitoring fish health and response to natural and anthropomorphic environmental change. Gill biopsies may also be useful for nonlethal sampling of fishes in these areas of research.

Materials and Methods

Fish

Juvenile Atlantic salmon (*Salmo salar*) of sea-run (undomesticated) parentage were obtained from the White River National Fish Hatchery (Bethel, VT, USA) and reared at the Anadromous Fish Research Center (Turners Falls, MA, USA) for several months prior to experiments. Fish were maintained in 1100-L tanks continuously supplied with fresh water (10°C) and aeration. Lighting was provided by overhead fluorescent lights and daylength was altered twice weekly to provide a simulated natural daylength. Seawater exposure was conducted in aerated 360-L tanks with continuous filtration through paper and charcoal.

Gill Biopsy

Gill filaments are removed while the fish are under anesthesia. A cartilaginous septum (present in some species, such as salmonids, but not others) holds filaments together for one half their length. Reflect back the operculum with rounded forceps. For 20–80 g fish, cut 4–6 filaments just above the septum with fine-point scissors (more or less tissue for smaller or bigger fish, respectively). Do not cut below the septum as this will cause substantial bleeding. As the tissue is cut, rotate scissors slightly so that filaments stay on the scissors. Be careful not to crush the sampled or remaining filaments. Immerse cut filaments in 100 μL of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) in a 0.5-mL microcentrifuge tube and freeze within 0.5 h. Gill tissue can be stored at −20°C for up to 6 wk and at −80°C for 3 mo.

Validation of Gill Biopsy

Juvenile Atlantic salmon weighing 44–77 g were anaesthetized and length and weight recorded. Twenty fish were marked with a visual implant tag (Northwest Marine Technology Inc., Shaw Island, WA, USA) posterior to the eye, and 10 of these received a gill biopsy. A group of untagged fish was included to ensure that the visual implant tag did not affect growth. Water temperatures were maintained at 12.5 ± 1°C throughout the experiment. After 26 d, fish were anaesthetized and length and

TABLE 1. Specific growth rate (weight), condition factor, gill Na^+, K^+-ATPase activity, and plasma sodium after seawater challenge in control or gill biopsy groups of juvenile Atlantic salmon. Values are mean ± SE (n = 10 per group). There was no significant difference between the groups in any of the measured parameters ($P > 0.2$, Student's t-test). This experiment was conducted in autumn when gill Na^+, K^+-ATPase activity is at its lowest level.

	Control	Gill biopsy
Growth rate (%·d^{-1})	0.57 ± 0.09	0.56 ± 0.09
Condition factor 100·(weight/length^3)	1.16 ± 0.02	1.15 ± 0.01
Gill Na^+, K^+-ATPase activity (μmol ADP·mg protein^{-1}·h^{-1})	0.72 ± 0.07	0.89 ± 0.10
Plasma sodium (mM) (after seawater challenge)	180 ± 2	176 ± 2

weight were recorded. Gill tissue was removed from all tagged fish for later measurement of gill Na^+, K^+-ATPase activity. Ten fish receiving their first gill biopsy and 10 fish without gill biopsy were kept in fresh water for 24 h and were then transferred to 25 ppt seawater (Clarke et al. 1985). After 24 h, fish were anaesthetized and blood removed from the caudal vessels into heparin-treated syringes. Blood was centrifuged at 5000g for 5 min and plasma removed and frozen at −80°C. Plasma sodium concentrations were measured by ion-selective electrodes (AVL Scientific Corp., Rosewell, GA, USA).

Measurement of Gill Na^+, K^+-ATPase Activity

Measurement of Na^+, K^+-ATPase activity is similar to that presented by Penefsky and Bruist (1984) and McCormick and Bern (1989), modified for 96-well microplates. The ouabain-sensitive hydrolysis of adenosine triphosphate (ATP) is enzymatically coupled to the oxidation of nicotinamide adenine dinucleotide (reduced form, NADH), which is directly measured in a microplate reader. An assay mixture (solution A) containing 4 U lactate dehydrogenase (LDH)·mL^{-1}, 5 U pyruvate kinase (PK)·mL^{-1}, 2.8 mM phosphoenolpyruvate (PEP), 0.7 mM ATP, 0.22 mM NADH, and 50 mM imidazole (pH 7.5) is made just prior to the assay and is stable for 2–3 d at 4°C (all biochemical reagents from Sigma, St. Louis, MO, USA). Assay solution B is as above but also contains 0.5 mM ouabain. A salt solution containing 189 mM NaCl, 10.5 mM $MgCl_2$, 42 mM KCl, and 50 mM imidazole (pH 7.5) is prepared in advance and is stable for several weeks at 4°C. Mix the assay solutions (A and B) and salt solutions separately in a 3:1 ratio, and keep on ice. A few minutes before use, place the amount of assay mixture to be used in a 25°C water bath. Before each day's use, run a standard curve from 0 to 20 nmol ADP·well.$^{-1}$ A decrease in NADH will occur immediately and should become stable within 2–3 min. The slope of the standard curve should be −0.019 to −0.020 OD unit·nmol ADP^{-1}·well^{-1}.

Thaw samples immediately prior to assay and keep on ice throughout; activity will begin to decrease immediately after homogenization, so activity measurements should begin within 0.5 h of tissue homogenization. Add 25 μL of SEID (0.5 g of sodium deoxycholate in 100 mL of SEI) to the microcentrifuge tube and homogenize filaments in the tube using a motorized pestle (Kontes, Vineland, NJ, USA). Grind tissue for 10–15 s, visually ensuring that all tissue (except cartilage) has been fully homogenized. Centrifuge at 5000g for 30 s to remove insoluble material.

With the microplate on an ice pack or other cold, dry surface, add 10 μL of homogenate to each of four wells for every sample. The remaining homogenate is removed and saved for later analysis of protein content (Smith et al. 1985). Add 200 μL of the A-plus-salt mixture to two wells per sample and 200 μL of the B-plus-salt mixture to the other two wells per sample. The plate is then placed in a temperature-controlled plate reader (we use Thermomax, Molecular Devices Corp., Menlo Park, CA, USA) and the linear rate of NADH disappearance is measured at 340 nm for 10 min. The linear rate from 2 to 10 min in each pair of duplicate wells is determined, and Na^+, K^+-ATPase activity is calculated as the difference in ATP hydrolysis in the absence and presence of ouabain, expressed as micromoles of ADP per milligram of protein per hour.

Results and Discussion

There were no mortalities after performing gill biopsies on juvenile Atlantic salmon in this experiment, or in several other experiments in which more than 100 fish were sampled.

There was no significant difference in growth rate of control fish and those receiving a gill biopsy in the 3-wk period following the procedure (Table 1). A third group that did not receive visual implant tags had the same average growth rate (0.54%·d^{-1}) as the tagged fish. In gill tissue taken from the same individuals, there was no difference in gill Na^+, K^+-ATPase activity initially (first biopsy) or after 26 d (second biopsy; $P > 0.05$, Student's t-test). Similarly, there was no difference in gill Na^+, K^+-ATPase activity in the control and gill biopsy groups at the end of 26 d (Table 1).

One day after receiving a gill biopsy, 10 control and 10 treated fish were exposed to a seawater challenge. There was no significant difference in the ability of the two groups to regulate plasma sodium after 24 h in 25 ppt seawater (Table 1).

Bleeding does not normally occur when using this method. Some blood loss may occur if the gill arch is mistakenly cut below the septum, but even this will not result in the death of the fish. The small portion of the total gill filament tissue that is removed in this method (0.3–0.6% in 50-g fish) makes it unlikely that gill functions of the fish will be greatly affected. This is supported by our inability to detect differences in growth rate or salinity tolerance after gill biopsy. However, there is obviously local tissue damage and temporary exposure of the blood to the external medium. Although we have not experienced any disease in fish with gill biopsies, this may be an important consideration, particularly in a hatchery setting. The cut surface heals within several days, but we have observed no significant regeneration of the filaments over a period of several months. Zaugg and McLain (1971) reported that coho salmon (*Oncorhynchus kisutch*) with approximately 10% of the gill filamental tissue removed lived for the duration of a 2-h experiment. Remarkably, Nanba et al. (1971) found that common carp (*Cyprinus carpio*) were able to survive removal of 75% of gill filament tissue (six of eight gill arches).

The method for measurement of Na^+, K^+-ATPase presented above is highly sensitive and reproducible. The coefficient of variation of a single homogenate was 4.7% (n = 6); variation of several gill biopsies from the same gill arch was 11.0% (n = 6). Na^+, K^+-ATPase activity increased linearly with increasing amounts of homogenate from 5 to 25 μL (4–20 μg of protein).

Incubation temperature for measurement of gill Na^+, K^+-ATPase activity in the present study was 25°C. This tempera-

TABLE 2. Gill Na^+, K^+-ATPase activity ($\mu mol \cdot mg\ protein^{-1} \cdot h^{-1}$) in Atlantic salmon presmolt (mid-February) and smolt (mid-May) and ratio (smolt:presmolt) using the method outlined in this paper and that of Zaugg (1982; data from McCormick et al. 1987). Values are mean ± SE of 10–12 fish per group.

	Presmolt	Smolt	Ratio
Present method	3.7 ± 0.5	15.2 ± 1.6	4.1
Zaugg (1982) method	16 ± 1	60 ± 5	3.8

ture was chosen because it is within the thermal limits of salmonids and most other teleosts and can be readily compared with other enzymes and metabolic processes measured at this temperature. Because gill Na^+, K^+-ATPase activity of salmonids increases linearly with increasing temperature up to 37°C, the activity of this enzyme can be measured with validity at these higher temperatures (and thereby increases the sensitivity of the assay). This may not be true, however, of all enzymes in a species, or for Na^+, K^+-ATPase in other species, and should be validated for each species.

Although the use of a crude homogenate results in lower specific activity than use of partially purified membrane preparations (Zaugg 1982), the relative changes in gill Na^+, K^+-ATPase activity between presmolts and smolts are identical (Table 2). Of the approximately fourfold difference in specific activity of the two methods, approximately 50% is due to the removal of soluble proteins in the partial membrane purification, and 50% is due to the higher temperatures (McCormick and Bern 1989). The use of crude homogenates has the advantage of losing only a small amount of total Na^+, K^+-ATPase activity during sample preparation (less than 10% in the present method, up to 30% as reported by Zaugg 1982). This advantage permits more direct comparison of Na^+, K^+-ATPase activity in other tissues and with other enzyme activities. Crude homogenates have the disadvantage of including soluble proteins that lower specific activity and may interfere with activity measurements. The comparisons of Table 2 and the successful use of this method in other studies (McCormick and Bern 1989; unpublished data) indicate that these are not major concerns in the study of gill Na^+, K^+-ATPase activity in salmonids.

There are other sensitive measures of gill Na^+, K^+-ATPase activity in fishes (e.g. Mayer-Gostan and Lemaire 1991), and it should be possible to modify these for use with gill biopsies. By modifying the salt mixture or inhibitors, these methods can be used to measure other ion-transporting ATPases (unpublished data; Mayer-Gostan and Lemaire 1991). After becoming proficient, the time required for the outlined method of gill biopsy is the same or less than that necessary for removing large pieces of gill tissue from the gill arch. No expensive equipment is required, although a pair of fine scissors is recommended. Although investigation of the parr–smolt transformation has been emphasized, these methods may be useful in research on pollution, disease, development, and environmental change in fishes.

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