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Acute temperature elevation in tap and Rhine water affects skin and gill epithelia, hydromineral balance, and gill Na^+/K^+ -ATPase activity of brown trout (*Salmo trutta*) smolts

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Abstract: The effects of a 3-h temperature elevation of 7°C were studied for 29 days on the brown trout (*Salmo trutta*) smolt in tap water and in water from the lower Rhine. The effects in the skin were apparent at 3 h and included depletion of electron-dense vesicles and increased numbers of heavily stained desmosomes in the filament cells of the upper epidermis. Increased levels of apoptosis and necrosis occurred and were associated with leukocyte infiltration of the epidermis. Similar effects in the gill epithelium were mainly confined to the chloride cells. Highest levels of necrosis in skin and gill epithelia occurred in fish that were temperature shocked in Rhine water. Effects of exposure to Rhine water alone were intermediate between those of temperature shock in tap water and in Rhine water. At 29 days, recovery was good in tap water, partial in Rhine water, and poor for the fish temperature shocked in Rhine water. Although disruption of hydromineral balance was not indicated in plasma electrolytes, specific Na^+/K^+ -ATPase activities in the gill were higher for all treatments at 24 h and for the groups temperature shocked in Rhine water at 8 days. Overall, temperature shock in Rhine water gives additive stress effects and poor recovery at 29 days.

Résumé : On a étudié durant 29 jours les effets d'une hausse de la température de 7°C d'une durée de 3 h sur des smolts de la truite brune (*Salmo trutta*) dans de l'eau du robinet et dans de l'eau du cours inférieur du Rhin. Les effets sur la peau étaient apparents après 3 h, dont une déplétion des vésicules à densité d'électrons élevée et un accroissement du nombre de desmosomes fortement colorés dans les cellules filamenteuses de l'épiderme supérieur. On a observé des niveaux accrus d'apoptose et de nécrose, associés à une infiltration de leucocytes dans l'épiderme. Les effets similaires observés dans l'épithélium des branchies étaient principalement limités aux cellules à chlorures. Les plus forts niveaux de nécrose dans la peau et l'épithélium des branchies ont été observés chez les poissons soumis au choc thermique dans l'eau du Rhin. Les effets de la seule exposition à l'eau du Rhin étaient intermédiaires entre ceux découlant du choc thermique dans l'eau du robinet et dans l'eau du Rhin. À 29 jours, le rétablissement était bon pour l'eau du robinet, partiel pour l'eau du Rhin et faible dans le cas des poissons soumis au choc thermique dans l'eau du Rhin. Bien que les électrolytes plasmatiques n'aient pas révélé l'existence d'une perturbation de l'équilibre hydrominéral, les activités spécifiques de la Na^+/K^+ -ATPase dans les branchies étaient accrues dans tous les traitements à 24 h et l'étaient encore à 8 jours chez les groupes soumis au choc thermique dans l'eau du Rhin. En résumé, le choc thermique dans l'eau du Rhin a été le plus stressant, et les poissons ne s'en étaient pas bien rétablis après 29 jours.

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Introduction

Fishes are ectotherms (i.e., having a primarily external source of body heat), and as such, their body temperature is dictated by the external environmental temperature. Because

the heat capacity of water is a factor of 3000 higher than that of air, thermal conductance of water is also higher, and therefore, fish are greatly influenced by the effects of increased temperature. Temperature affects all levels of biological organization, and considerable literature exists reporting the effects of temperature on fishes (e.g., Hazel 1993; Goldspink 1995). The impact of short-term acute increases in temperature is a serious concern for fish populations, and migrating salmonids are especially sensitive to acute temperature increases (Coutant 1973; Cherry et al. 1975; Gray 1990). Although the role of acute temperature shock has been studied and reported from a number of laboratory experiments, the effects of thermal discharges on free-moving juvenile salmonids have not been determined (Gray 1990).

Thermal discharges from anthropogenic water use (e.g., cooling waters for industry) threaten fish both directly through temperature shock (Coutant 1973; Cherry et al.

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1975; Gray 1990) and impingement at intake pipes (Hadderingh et al. 1983) and indirectly through interactive effects with pollutants (Reid et al. 1997) and alterations to thermal microhabitats (Spigarelli and Thommes 1979; Haynes et al. 1989). Thus, temperature shock alone may be considered a physical stressor, which in combination with chemical stressors, such as pollutants, may increase the toxicity. This may occur either by temperature directly affecting the physicochemical properties of the pollutants or by the additive effects of temperature stress and pollution stress.

The Rhine, the largest and most important European river, has been affected by a variety of anthropogenic factors, including both thermal and xenobiotic pollution (for overviews, see Friedrich and Muller 1984; Van Dijk et al. 1995). The Rhine had self-sustaining salmon populations historically, but these have dramatically declined (Cazemier 1994; Van Dijk et al. 1995). Because salmonids have high water quality requirements and the anadromous lifestyle results in the utilization of the whole river system, the Atlantic salmon (*Salmo salar*) has been chosen as the indicator species whose successful reestablishment confirms the improved water quality and health of both the Rhine and its catchment.

Recent international negotiations have targeted improving the quality of the water by agreements on reducing levels of contaminated discharges into the river (Schulte-Wulver-Leidig 1994). However, thermal pollution is a stress factor that is often ignored, and there is a thermal gradient from the upper to the lower Rhine, resulting from the utilization of river water for cooling water by industry. Migrating salmonids encounter a thermal gradient, as well as numbers of thermal plumes, as they migrate through the river. In the Dutch part of the lower Rhine, cooling waters are discharged by industry at 7°C above intake temperature. A single acute 3-h temperature shock of +7°C induces prolonged stress-related effects in the skin of freshwater rainbow trout (*Oncorhynchus mykiss*) (Iger et al. 1994b), but it is not yet known to what extent this may disrupt other functions, such as hydromineral balance or gill function in freshwater.

While Atlantic salmon have been absent from the Rhine for many years, the brown trout (*Salmo trutta*) (anadromous) has always been present in some numbers (Cazemier 1994). Rainbow trout and brown trout skin responds to Rhine water with a number of ultrastructural changes very similar to those shown by fish stressed under laboratory conditions (Iger et al. 1994b, 1995), suggesting that salmonid populations in the river system may be stressed (Iger et al. 1994c; Nolan et al. 1998). Many of the changes reported in the skin of the fish are mediated by cortisol (Iger et al. 1995), which is the main corticosteroid in teleosts and is known to be hypersecreted during stress (Wendelaar Bonga 1997). Together, these data indicate that exposure to present-day Rhine water evokes a stress response, at least at the level of the skin epithelium. Any interactions between temperature and the pollutants in Rhine water have not been reported to date.

In the present study, we exposed the native brown trout smolt to a single acute 3-h temperature shock of +7°C in both tap water (TW) and water from the lower Rhine (RW) and sampled at 3 h and 1, 8, 18, 22, and 29 days to evaluate the effect of short-term acute temperature shock, such as would be experienced during migration through a single thermal plume. These sample points were chosen based on

the results from previous studies with rainbow trout and brown trout (Iger et al. 1994b, 1994c; Nolan et al. 1998). The fish were continuously exposed to water from the lower Rhine during the final stages of smoltification, which is the time that they are exposed to this river section in nature. In this way, we studied the effects of a temperature shock (a physical stressor) and RW (a chemical stressor) on the brown trout smolt. We looked for interactive effects of temperature with RW by combining the two stressor types. Because skin and gills are affected by toxicant exposure, we combined ultrastructural analysis of skin and gills with examination of plasma ions and gill Na⁺/K⁺-ATPase activity. These parameters have been validated as stress parameters connected with toxicity in earlier papers by our group (Wendelaar Bonga and van der Meij 1989; Iger et al. 1994b, 1994c, 1995; Nolan et al. 1998, 1999a, 1999b) and by other groups (Jagoe et al. 1996; Burkhardt-Holm et al. 1997; Kakuta 1997; Lionetto et al. 1998).

Materials and methods

Experimental setup

Presmolt brown trout (weight 39.37 ± 6.16 g, fork length 15.55 ± 1.02 cm) bred from a brown trout population naturally migrating from the North Sea into streams in Schleswig Holstein, Germany, were obtained in spring. The fish were placed in groups of 30 in 400-L black plastic tanks in running nonchlorinated TW with a flow-through rate of 600 L·h⁻¹. Each tank was strongly aerated by an air compressor via an airstone and mechanically filtered by an Eheim 2213 external power filter, increasing circulation by a further 440 L·h⁻¹. The fish were fed a standard pelleted trout diet at the rate of 1% of body weight daily. Lighting was controlled by a time switch, matched the natural photoperiod (February–April), and was adjusted periodically during the experiment. Windows in the laboratory meant that the fish could perceive changes in natural photoperiod.

After 3 weeks of acclimation, the water supply to four groups was changed from TW to RW. This was achieved with minimum disturbance to the groups by changing the inlet water supply pipe to these tanks from TW to RW at the source. The RW was pumped up at KEMA (near Arnhem, The Netherlands) and filtered by a lamellar filter system in a sediment chamber to remove particles larger than 2 mm (Iger et al. 1994c). The composition for some of the major contaminants in the RW during the exposure is given in Table 1 from data measured by RIZA near Lobith, The Netherlands, during the experimental period. Two groups of fish remained in TW as controls. The water temperature in two TW and two RW groups was then increased to 7°C above ambient for 3 h (TW&T and RW&T, respectively). This temperature shock was delivered by shutting off the flow-through system and raising the water temperature over a 30- to 50-min period using heating coils until a 7°C elevation was achieved (Iger et al. 1994b). The groups that were not temperature shocked were sham treated by shutting off the flow-through system for the same duration as for the other groups. As the TW and RW temperatures were different (9 and 12°C, respectively), the temperature shock increased water temperatures to 16 and 19°C, respectively. These temperatures were monitored and maintained for a 3-h period, after which the flow-through system was reopened, and the temperature returned to initial values within 60 min. The fourth treatment comprised two groups maintained continuously in RW.

The temperature profile for both RW and TW was measured daily. RW temperature was higher than TW temperature at the beginning, and both increased over time (TW temperature was 8°C at the beginning of the experiment and 11°C at the end, while RW

Table 1. Chemical parameters measured in Rhine water at Lobith during the experimental exposure of brown trout smolts.

Parameter	Rhine water range during experiment	Tap water values
General parameters		
pH	7.62–7.91	8.14
O ₂ (mg·L ⁻¹)	9.8–11.1	4.9
NO ₂ ⁻ (µg·L ⁻¹)	50.0–70.0	<20.0
NH ₄ ⁺ (µg·L ⁻¹)	100–180	<50
PO ₄ ⁻ (µg·L ⁻¹)	48–67.0	<30.0
Heavy metals (µg·L ⁻¹)		
Al	211.0	<5.0
Ba	88.0	20.5
Fe	1270–1980	30
Cd	0.05–0.11	<0.02
Cr	4.2–7.5	<1.0
Cu	6.0–8.2	<5.0
Hg	0.02–0.03	<0.05
Mn	97.0	<10.0
Ni	4.1–4.7	<5.0
Pb	3.8–9.6	<5.0
Zn	30.0–105.0	<5.0
Other ions (mg·L ⁻¹)		
Ca ²⁺	67–77.0	34.0
Cl ⁻	78–114.0	24.0
K ⁺	4.4–5.1	2.4
Na ⁺	46–67.0	15.6
Mg ²⁺	10.3–10.6	9.9

temperature was 12 and 17°C, respectively). Dissolved oxygen content of the water was checked daily and varied between 8.7 and 11.5 mg·L⁻¹ in TW and between 8.7 and 10.7 mg·L⁻¹ in RW during the experimental period.

Sampling

Fish were sampled at 3 h and at 1, 8, 18, 22, and 29 days after the beginning of the treatments. All sampling took place first thing in the morning before feeding. Four fish per treatment were sampled at each time point. Replicate tanks were sampled alternately on consecutive sample days, such that no tank was disturbed twice within the same week, and data are from replicate treatment tanks.

At sampling, skin for transmission electron microscopy and light microscopy was sampled first. Blood was withdrawn by needle into Na⁺-heparinized syringes from the caudal blood vessels. The fish were killed by spinal transection at the base of the skull, and length and weight data were collected to calculate condition factors for each fish. Heparinized blood was spun for 3 min in a bench centrifuge and plasma was pipetted off, aliquoted, and stored at -20°C. For osmolality and ion analysis, a Roebling cryoscopic microosmometer and a flame photometer (Technikon model IV autoanalyzer) were used. For determination of Na⁺/K⁺-ATPase activity, gill filaments were trimmed from the gill arches, rinsed and placed in ice-cold buffer (0.3 M sucrose, 20 mM Na₂EDTA, 0.1 mM imidazole, pH 7.1 with HCl), frozen immediately in liquid nitrogen, and stored at -80°C until analysis. The specific Na⁺/K⁺-ATPase activity was determined in an H₀ homogenate fraction by the method routinely used in our laboratory (Flik et al. 1984).

For transmission electron microscopy, samples were taken at 3 h and 1 and 29 days only to study immediate effects and long-term effects. From each fish, a piece (2 × 3 mm) of skin from the scaleless dorsal part of the head, along with a small piece of the first gill arch, were fixed in 3% glutaraldehyde buffered in sodium cacodylate (0.09 M, pH 7.3) on ice for between 15 and 30 min and postfixed for 1 h on ice with 1% osmium tetroxide and 2.5% potassium bichromate in the same buffer (Wendelaar Bonga and van der Meij 1989). Ethanol-dehydrated tissues were embedded in Spurr's resin, after which ultrathin sections of skin and gill were cut by diamond knife and collected on a 150-mesh copper grid. Four grids per sample were prepared; these were stained with uranyl acetate and lead citrate before viewing in a Philips EM 300 transmission electron microscope at 40 kV. Overall condition of the epithelia was examined and 10 representative views per specimen were photographed by an analyst without knowledge of the treatment groups involved.

In the skin, five areas from the upper epidermis of each fish were scanned and photographed at low magnification; the negatives were enlarged four times and printed. From these micrographs, numbers of electron-dense vesicles and electron-dense desmosomes were quantified in 7–10 filament cells per fish (each filament cell representing a cytoplasmic area of about 250 µm²) and averaged to give mean values per fish. Care was taken to include data only from mature filament cells with a visible nucleus, which were neither necrotic nor apoptotic. Overall ultrastructure of the epidermis was examined, and particular attention was paid to cellular necrosis (characterized by nuclei with aggregations of chromatin, swelling of the cytoplasmic compartment, and loss of apical microridges) and apoptosis (characterized by progressive densification of the nucleus, organelles, and cytoplasm, leading to shrinkage and loss of contacts with surrounding cells), as well as leukocyte infiltration and epithelial integrity (evaluated by intercellular swelling and cell-cell contacts). For gill specimens, attention was focused on the interlamellar parts of the filaments where the chloride cell populations are located, and necrosis (defined above), apoptosis (characterized for chloride cells by progressive densification of the mitochondria, nucleus, and cytoplasm as well as dilation of the tubular system), and leukocyte infiltration were evaluated. From the micrographs, the tissues were evaluated and scored semiquantitatively against TW controls using a graded evaluation system (-, +, ++, +++) to represent incidence of each parameter.

For light microscopy, skin samples (5 × 5 mm) were fixed in Bouin's fixative and processed to 5-µm sections by conventional methods. Replicate series of sections were stained with either periodic acid Schiff's or Alcian Blue (pH 2.5) to identify total and acidophilic mucous cells, respectively. The numbers of mucous cells were quantified using a calibrated brightfield microscope, as described in Nolan et al. (1999b).

Data handling and statistics

For statistical analysis, raw data (except condition factor and Na:Cl ratio) were log transformed and effects of treatments were analyzed by one-way ANOVA at each sample point. Differences between treatments were assessed by a Tukey-Kramer multiple comparisons test. For analysis of control values of gill Na⁺/K⁺-ATPase activity over time, one-way ANOVA of log-transformed data was applied and trend analysis of raw data to 18 days was applied to demonstrate the presence of a linear trend. For the parameters condition factor and Na:Cl ratio, differences between controls and treatments as well as in controls over time were assessed with the Mann-Whitney *U* test. In each case, data are presented as means ± SEM for a sample size of *n* = 4. Statistical significance was accepted at *P* < 0.05.

Results

Fish health and smolt status

Specific gill Na⁺/K⁺-ATPase activity and condition factor of control fish were used to indicate smolt status. Gill

Fig. 1. Gill Na^+/K^+ -ATPase activity ($\mu\text{mol ATP}\cdot\text{mg protein}^{-1}\cdot\text{h}^{-1}$) (black bars) and condition factor profiles (gray bars) for control brown trout smolts maintained in tap water during the experimental period. * $P < 0.05$ compared with 3- and 24-h values.

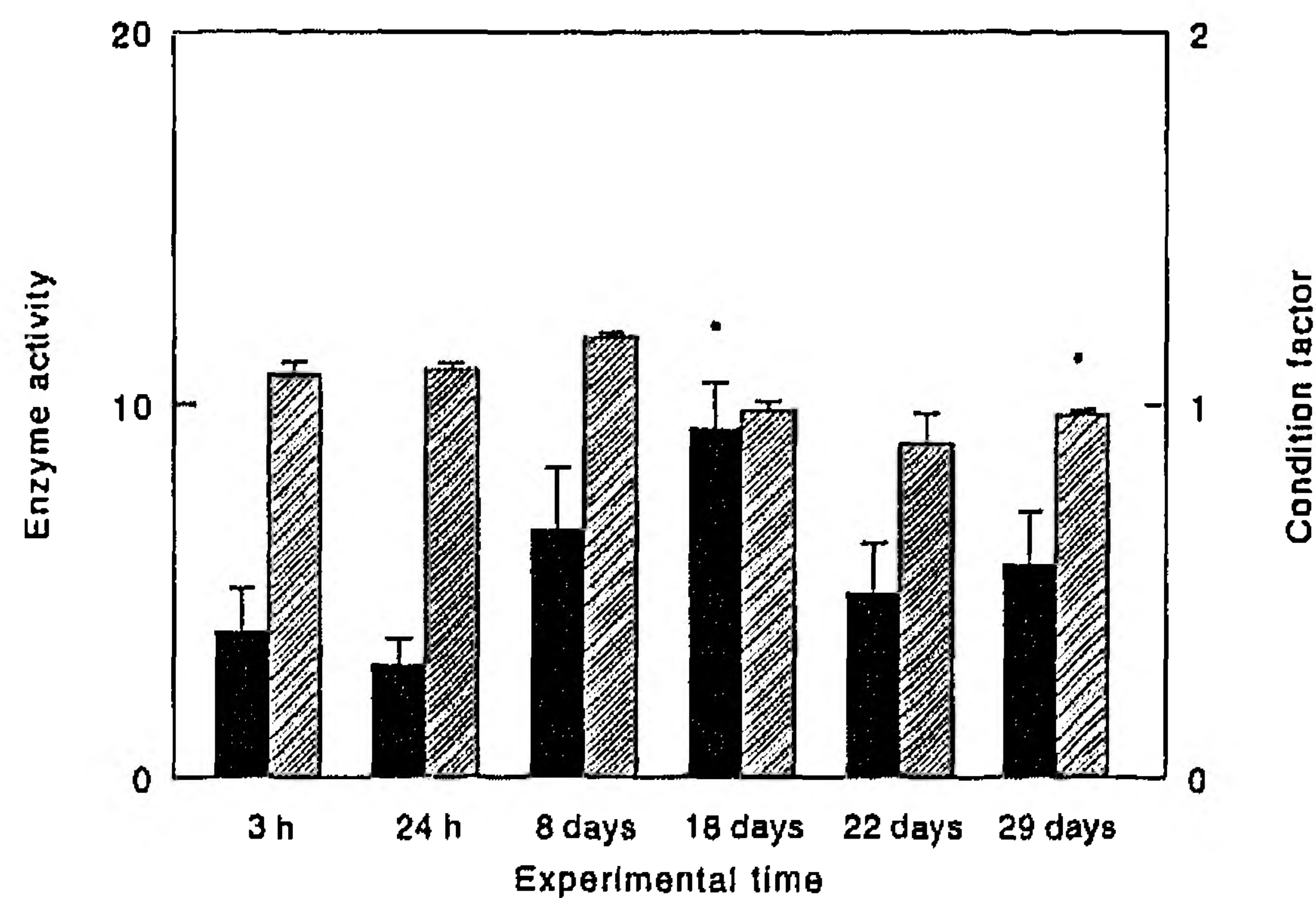


Table 2. Gill Na^+/K^+ -ATPase activity in brown trout smolts in tap water and following exposure to Rhine water, a single 3-h temperature shock of $+7^\circ\text{C}$ in tap water (TW&T), or the combination of the two (RW&T).

Sampling point	TW	TW&T	RW	RW&T
3 h	3.9±1.14	4.9±1.23	5.8±0.89	8.4±1.54
24 h	3.0±0.72	7.9±0.70**	8.1±1.64**	8.5±1.26**
8 days	6.6±1.65	6.7±1.03	8.3±1.40	14.47±2.69*
18 days	9.3±1.27	10.5±2.33	7.8±0.87	2.3±1.29**
22 days	4.9±1.35	10.1±2.54	6.7±0.98	5.6±0.70
29 days	5.7±1.44	6.2±2.88	4.2±1.39	3.43±0.26

Note: Data are presented as means \pm SEM for a sample size of $n = 4$ and expressed in units of enzyme activity ($\mu\text{mol P}_i\cdot\text{mg protein}^{-1}\cdot\text{h}^{-1}$). *Significantly different from TW control at $P < 0.05$; **significantly different from TW control at $P < 0.01$.

Na^+/K^+ -ATPase activity of TW fish increased to 18 days, indicated by trend analysis (slope 2.471, $r^2 = 0.4732$, $F = 7.876$, $P = 0.0038$), while condition factor decreased beyond this point (Fig. 1). Condition factors of the different treatments did not differ from those of TW fish at any point. All fish fed well throughout the period and remained in good condition and disease free. Overall mortality was less than 1.5%, evenly distributed over all treatments.

Plasma ions

Plasma osmolality, sodium, and chloride were not significantly different between treatment groups at any sample point and were in the range considered normal for freshwater salmonids (data not shown).

Gill Na^+/K^+ -ATPase activity

Specific gill Na^+/K^+ -ATPase activity (Table 2) was unaffected by the treatments at 3 h and was higher than in controls in the TW&T, RW, and RW&T groups at 24 h. Specific enzyme activity in the RW&T treatment was also significantly higher than in controls at 8 days and significantly

Table 3. Ultrastructural effects in the skin and gill epithelia of brown trout smolts in tap water and following exposure to Rhine water, a single 3-h temperature shock of $+7^\circ\text{C}$ in tap water (TW&T), or the combination of the two (RW&T).

	TW	TW&T	RW	RW&T
Skin parameter				
Overall apoptosis	+	++	+++	+++
Overall necrosis	+	++	+++	+++
Intercellular spaces	-	+	++	+++
Electron-dense vesicles	+++	++	+	+
Filament cell desmosomes	+	+++	+++	+++
Macrophage and leukocyte infiltration	+	++	+++	+++
Epidermal recovery	-	Good	Partial	Poor
Gill parameter				
Overall apoptosis	+	+	+	+
Overall necrosis	+	+	+	+
Intercellular spaces	-	+	++	+++
Chloride cell apoptosis	+	+	++	++
Chloride cell necrosis	-	+	++	+++
Chloride cell atrophy	-	-	+	++
Macrophage and leukocyte infiltration	+	++	+++	+++
Epithelial recovery	-	Good	Partial	Poor

Note: See the text for details of the parameters and evaluation methods used.

lower at 18 days. Na^+/K^+ -ATPase activity was similar in all treatment groups at 22 and 29 days.

Light microscopy of skin

Total numbers of mucous cells and acidophilic mucous cells were not significantly different between treatment groups at any sample point (data not shown).

Electron microscopy of skin

The skin of TW control smolts generally agreed with that described previously for trout (Iger et al. 1994c; Burkhardt-Holm et al. 1997; Nolan et al. 1998). The uppermost layer of cells is differentiated into specialized pavement cells that have apical microridges (Fig. 2A). Many microfilament-containing cells below the pavement cell layer were highly active, synthesizing electron-dense vesicles (Fig. 2A). These cells were interconnected by desmosomes, and little intercellular spacing occurred (Table 3; Fig. 2A). Occasional sloughing of pavement cells was observed, and necrotic and apoptotic cells were seldom seen in the inner cell layers (Table 3). At 29 days, the epidermal structure of TW control fish was similar except for the reduction in the numbers of electron-dense vesicles to minimal values (Figs. 3A and 4).

TW&T resulted in disruption of the pavement and filament cells at 3 h. Sloughing necrotic pavement cells, characterized by nuclei with aggregations of chromatin, swelling of the cytoplasmic compartment, and loss of apical microridges, were frequently observed (Fig. 2B). Populations of electron-dense vesicles in the filament cells were reduced at 3 and 24 h after temperature shock (Fig. 3A). The number of desmosomes per filament increased at 3 and 24 h (Fig. 3B). Leukocytes and macrophages were more frequently encoun-

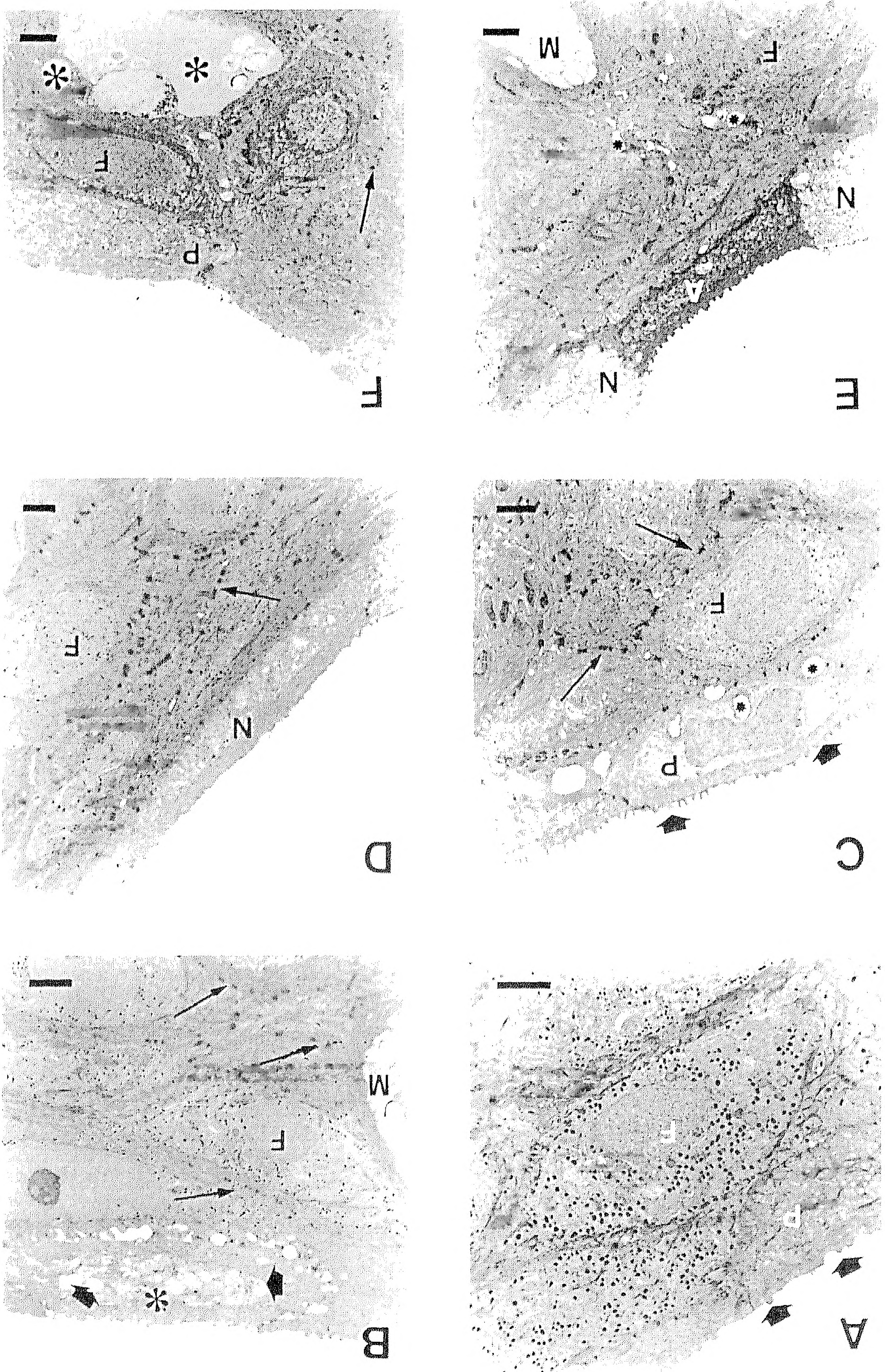


Fig. 2. (A) Upper epidermis of skin from control brown trout at the 3-h sample point. The pavement cells (P), showing microridges at the upper cell limits (arrows), form the interface with the water. The epithelium has good structural integrity. Filament cells (F) contain substantial amounts of electron-dense vesicles. Scale bar = 5 μ m. (B) Upper epidermis of skin from brown trout after a 3-h temperature shock of +7°C in tap water. A pavement cell is detaching (asterisk), showing swollen, electron-lucent organelles (thick arrows) and shrinkage characteristic of upper epidermal layer cells. This cell was in the process of sloughing off. The epithelium has good structural integrity, although numbers of electron-dense vesicles in the filament cells are lower than in unshocked fish. Heavily stained desmosomes become more obvious (thin arrows). M, mucous cell. Scale bar = 5 μ m. (C) Upper epidermis of skin from brown trout at 29 days after a 3-h temperature shock of +7°C in tap water. Pavement cells have basally located intercellular vacuolation (asterisks) and good apical microridge structures (thick arrows). The epithelium has good structural integrity overall, although numbers of electron-dense vesicles in the filament cells are much lower than previously. Heavily stained desmosomes are clearly seen (thin arrows). Scale bar = 5 μ m. (D) Upper epidermis of skin from brown trout after 3 h in Rhine water. The pavement cell layer is necrotic (N), and cells have swollen, electron-lucent organelles and cytoplasm and the apical microridge structures are gone. Although the epithelium below has good structural integrity, intercellular spaces are opening up and numbers of electron-dense vesicles in the filament cells are greatly depleted. Heavily stained desmosomes are already very obvious (arrow). Scale bar = 5 μ m. (E) Upper epidermis of skin from brown trout after a 3-h temperature shock of +7°C in Rhine water. The pavement cell layer is composed of both necrotic and apoptotic (A) cells. The epithelial structural integrity is compromised already as intercellular spaces are opening up (asterisks). Numbers of electron-dense vesicles in the filament cells (F) are greatly depleted, and heavily stained desmosomes are already clearly seen. Scale bar = 5 μ m. (F) Upper epidermis of skin from brown trout 29 days after a 3-h temperature shock of +7°C in Rhine water. The pavement cell layer is swollen and all cells contain many electron-lucent vesicles. The epithelial structural integrity is compromised still, and large intercellular spaces are present (asterisks). Electron-dense vesicles in the filament cells are barely discernible, and heavily stained desmosomes are still clearly visible (arrow). Scale bar = 5 μ m.

Fig. 3. Numbers of (A) electron-dense vesicles (EDV) and (B) desmosomes per filament cell from the head skin of brown trout smolts at 3 and 24 h posttreatments. TW control, tap water control; TW&T, after a 3-h temperature shock of +7°C in tap water; RW, Rhine water; RW&T, after a 3-h temperature shock of +7°C in Rhine water. * P < 0.05, ** P < 0.01, and *** P < 0.001 compared with control values.

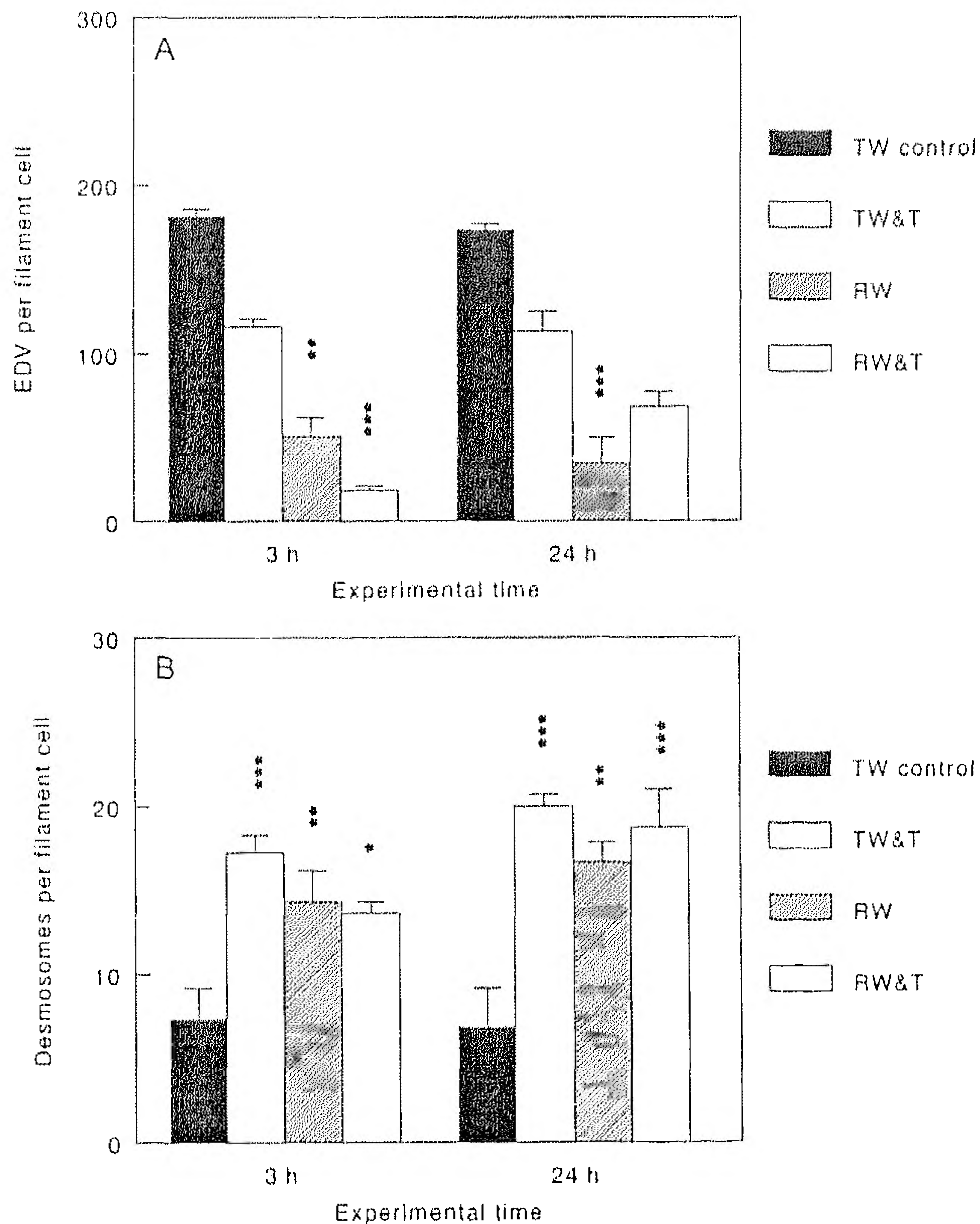
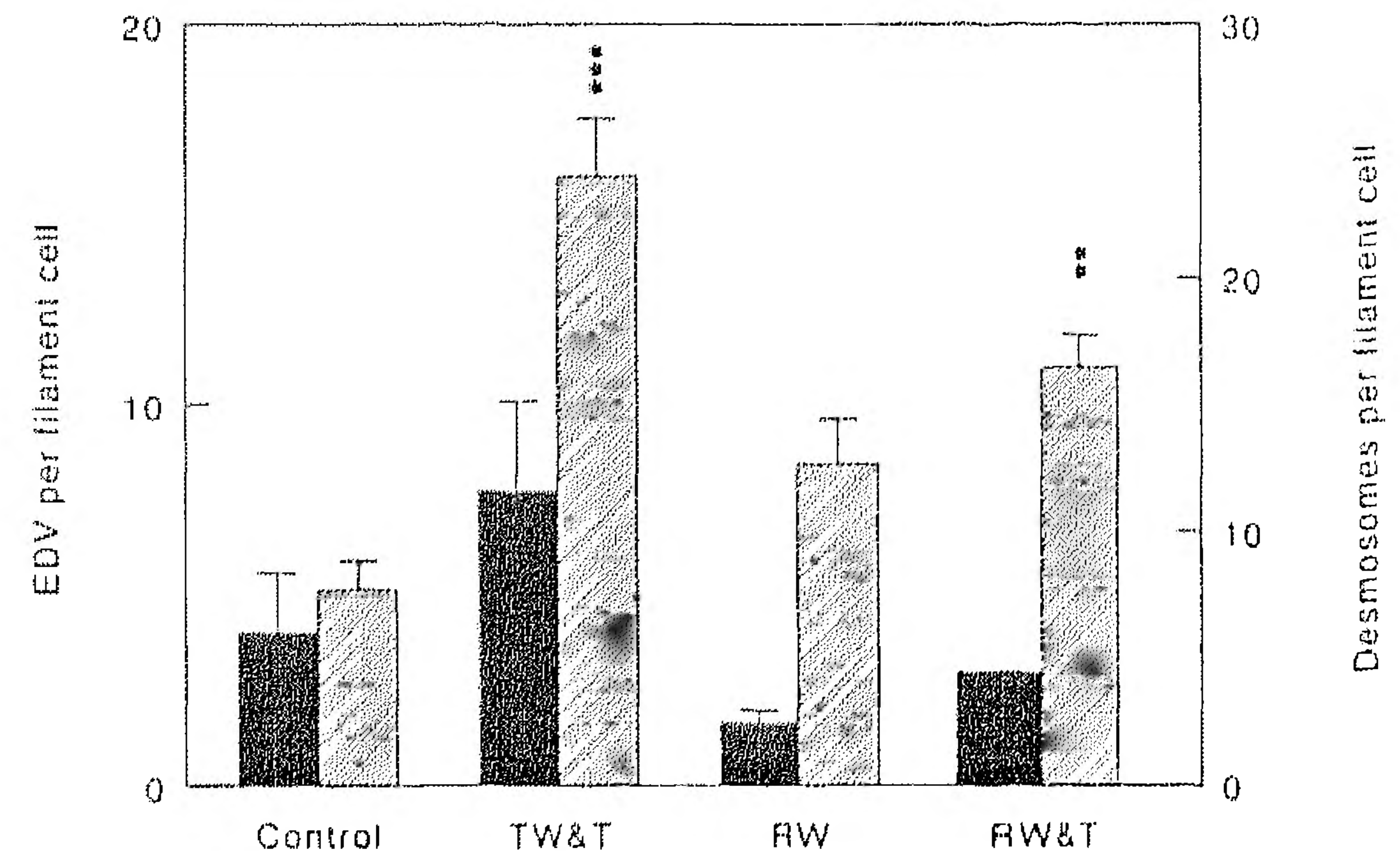


Fig. 4. Numbers of electron-dense vesicles (EDV) (black bars) and desmosomes (gray bars) per filament cell from the head skin of brown trout smolts at 29 days posttreatment. TW control, tap water control; TW&T, after a 3-h temperature shock of +7°C in tap water; RW, Rhine water; RW&T, after a 3-h temperature shock of +7°C in Rhine water. ** P < 0.01 and *** P < 0.001 compared with control values.



tered than in TW controls (Table 3). At 29 days, the epidermal condition of the TW&T fish had improved, although intercellular spaces still occurred (Fig. 2C; Table 3). Numbers of filament cell electron-dense vesicles were similar to those in the TW controls (Fig. 4), while numbers of desmosomes remained elevated (Fig. 4). At 29 days, lymphocytes and macrophages were comparable with those in the TW controls (Table 3).

Exposure to RW increased apoptosis and necrosis in the filament cell and pavement cell layers within 3 h. Necrosis of the pavement cells, characterized by swollen organelles and cytoplasm and subsequent membrane rupture, was a prominent feature (Fig. 2D). Numbers of electron-dense vesicles in the inner filament cells were reduced at 3 and 24 h (Fig. 3A). Intercellular spaces were opening at 3 h (Fig. 2D), and numbers of filament cell desmosomes were higher than in TW fish at 3 and 24 h (Fig. 3B). Macrophages and lym-

Fig. 5. (A) Representative transmission electron micrograph of the interlamellar region of the gill from control brown trout at the 3-h sample point. Many mitochondria-rich chloride cells (C) are present and have apical microstructures (arrows). There are few enlarged intercellular spaces, epithelial integrity is good, and some leukocytes (L) can be seen. E, erythrocytes; B, blood space; W, water. Scale bar = 10 μm . (B) Interlamellar region of the gill from brown trout at 24 h after a 3-h temperature shock of +7°C in tap water. A macrophage (asterisk) is engulfing an atrophying mitochondria-rich chloride cell that is still in contact with the water apically (arrow). The result is the development of intercellular spaces (S). Scale bar = 5 μm . (C) Basal lamellar region of the gill from brown trout after 3 h in Rhine water. The structural integrity of the tissue is very disrupted, and intercellular lymphatic spaces have opened up (asterisks), contributing to lamellar swelling. Chloride cells are atrophying in a manner characteristic of early apoptosis (AC). Many macrophages are in the epithelia and several contain phagocytosed material in lysosomes (arrow). Scale bar = 10 μm . (D) Lamellar region of the gill from brown trout after 29 days in Rhine water. Atrophying chloride cells are commonplace, although the atrophying is not always characteristic of early apoptosis. Here, the organelles are not swollen, although the cell has lost apical contact with the water. Scale bar = 5 μm . (E) Basal interlamellar region of the gill from brown trout after a 3-h temperature shock of +7°C in Rhine water. The structural integrity of the tissue is very disrupted and many intercellular lymphatic spaces have opened up (asterisks), contributing to lamellar swelling. Chloride cells are degenerating by apoptosis (arrow) and necrosis (NC). Scale bar = 10 μm . (F) Necrotic chloride cells in the gill from brown trout 24 h after a temperature shock of +7°C in Rhine water. Chloride cells necrosis is indicated by swollen cells and organelles. The membranes are ruptured, the cytoplasm is electron lucent, and, in the late stages, cytoplasmic leakage occurs (arrows). Macrophages (M) are often associated with chloride cells. Scale bar = 5 μm .

phocytes were commonly found throughout the epidermis from 24 h onwards. At 29 days, the epidermis of RW fish showed slight improvement (Table 3). The electron-dense vesicles in the filament cells were virtually absent, and large areas of epithelial disruption were evident. Sloughing pavement cells and apoptosis in the inner cell layers were common (Table 3). Numbers of electron-dense desmosomes per filament cell were similar to those in TW controls at 29 days (Fig. 4). Macrophages and lymphocytes were less frequently observed than in the earlier stages but were more abundant than in control fish (Table 3).

RW&T increased the incidence of apoptosis and necrosis of pavement and filament cells. Both types of cell death were seen at 3 h and intercellular spaces were present (Fig. 2E). Populations of electron-dense vesicles in the filament cells were reduced compared with TW controls at 3 and 24 h (Fig. 3A). Numbers of filament cell desmosomes were higher at 3 and 24 h (Fig. 3B). Lymphocytes and macrophages were frequently seen. At 29 days, the epidermis of RW&T smolts had not recovered (Table 3). Necrotic pavement cells were common and intercellular spaces still occurred (Fig. 2F). At 29 days, filament cell electron-dense vesicle numbers were similar to those in TW controls, while numbers of desmosomes were higher (Fig. 4). Macrophages and lymphocytes were commonly seen throughout the epidermis (Table 3).

Electron microscopy of gill

The gill of the FW brown trout smolt is composed of a series of filaments with pairs of lamellae branching off alternately on both sides. The branchial epithelium is composed primarily of squamous epithelial cells, similar to the pavement cells of the epidermis, as well as mucous cells and chloride cells. Under control conditions, populations of chloride cells are located mainly in the interlamellar epithelium of the filament (Fig. 5A). Lymphocytes and macrophages are especially common in the gill filaments of brown trout (Table 3).

TW&T fish gills were not dramatically affected by the treatment. Some disruption of the epithelia was evident from intercellular spaces and infiltration by macrophages and lymphocytes (Fig. 5B). Although some swollen, necrotic

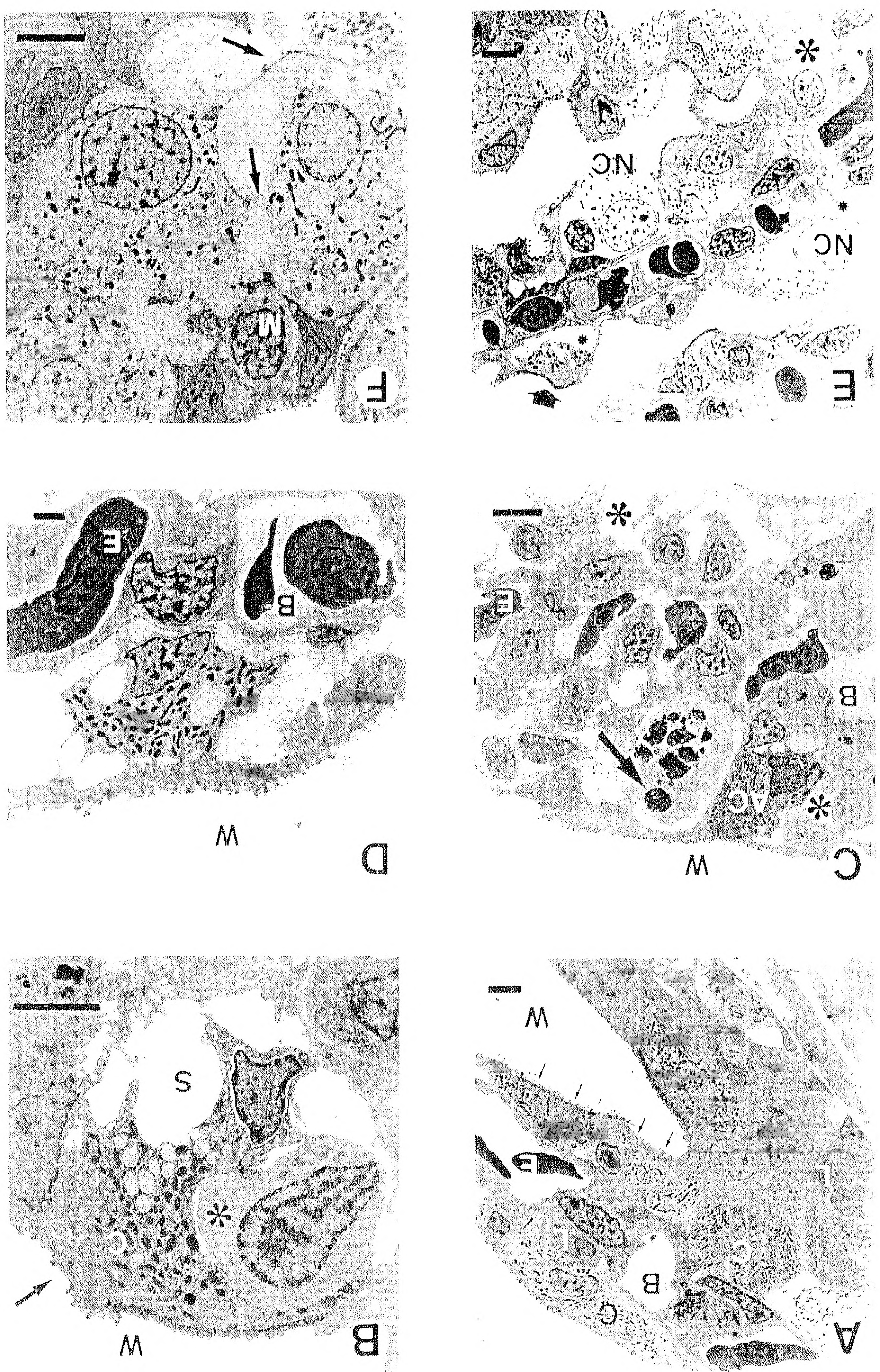
chloride cells were observed, apoptotic chloride cells were uncommon. At 29 days, the gill disruption was comparable with that in TW control fish (Table 3).

Exposure to RW resulted in alterations in the gill epithelia within 3 h (Fig. 5C). Intercellular spaces had opened up and were invaded by many macrophages and lymphocytes (Fig. 5C; Table 3). Necrosis, or sloughing of the superficial epithelial cells, was not observed. Necrotic chloride cells, characterized by swollen organelles and cytoplasm as well as aggregated chromatin, were commonly seen. Apoptotic chloride cells, identified by progressive densification of the cytoplasm and organelles and dilation of the tubular system, were frequently observed. Many leukocytes contained apoptotic fragments and lysosomes with cell debris (Fig. 5C). Some chloride cells appeared normal at the apical pole but atrophied at the basal pole, lost connection with surrounding cells, and were frequently associated with macrophages at this time (Fig. 5C). At 29 days in RW, the condition of the gills in these fish was improved (Table 3). There was a reduction in intercellular spaces, although atrophying chloride cells were still common (Fig. 5D).

RW&T treatment resulted in considerable disruption of epithelial integrity. At 3 h, substantial intercellular spaces opened up (Fig. 5E) and were infiltrated by lymphocytes and macrophages (Table 3). Necrotic chloride cells were commonly seen at 3 h (Fig. 5E) and at 24 h and 29 days. The swollen chloride cells were clearly necrotic and showed ruptured membranes and cytoplasmic leakage at late stage (Fig. 5F). Apoptotic and atrophied chloride cells were common and persisted through to 29 days. Lymphocytes and macrophages remained common at 29 days. The condition of the gill of the RW&T fish at this time was poor (Table 3).

Discussion

The results of this study show that short-term, sublethal, and acute temperature shocks, approximating the actual situation encountered by migrating salmonids in nature, induce effects in the brown trout smolt that may be considered stressful and that may have deleterious effects on the health status of the fish in nature. The behavioral effects of acute heat shock are severe and have been reported in terms of



increased susceptibility to predation in both stenothermic salmonids (Coutant 1973) and eurythermal cyprinids (Webb and Zhang 1994), indicating that temperature shock effects are severe for both thermosensitive and thermotolerant species alike. In the present study, we have demonstrated effects of acute temperature shock on the skin and gill epithelia and gill Na^+/K^+ -ATPase activity immediately and at 29 days posttreatment. For migrating smolts in nature, we speculate that these effects could lead to increased disease susceptibility or poor seawater adaptation.

The effects of temperature elevation in TW in the gill are less profound than effects on the skin. Both tissues recovered reasonably well within the 29-day period studied. Previously, prolonged effects over 14 days have been reported for a comparable temperature treatment in the skin of rainbow trout (Iger et al. 1994b). The present study shows a similar response in the skin and gill epithelia of the brown trout smolt and further indicates biochemical gill responses in terms of increased gill Na^+/K^+ -ATPase activity, while hydromineral balance is maintained. There are few studies where epithelial integrity, ion-transporting ATPases, and plasma electrolytes have been studied together, but our results corroborate those of other studies that report changes in ionoregulatory Na^+/K^+ -ATPases without electrolyte disturbance, e.g., after ectoparasitic infection of Atlantic salmon in seawater (Nolan et al. 1999b) and confinement stress in tilapia (*Oreochromis mossambicus*) in both freshwater and seawater (Nolan et al. 1999a). Many freshwater stressors induce ionoregulatory disturbance that, eventually, may result in a decrease in plasma electrolyte levels (Wendelaar Bonga 1997). The results of the current study point to the successful adaptation of the ionoregulatory mechanisms of the gills in response to an ionoregulatory challenge associated with the temperature shock, even when ultrastructure indicates poor condition. These transient changes in gill ATPase may reflect adjustments in ionoregulatory function but also extensive apoptosis of the chloride cells (an energy-dependent process indicating increased ageing of the cell population) and their replacement by newly differentiated cells. Similar effects have been reported in seawater-adapted Atlantic salmon postsmolts experimentally infected with numbers of sea louse (*Lepeophtheirus salmonis*). Here, measurements of plasma Na^+ and Cl^- indicated little effect on ionoregulation, while disrupted skin and gill epithelia suggested otherwise. This was explained by higher gill Na^+/K^+ -ATPase activity that was associated with increased chloride cell turnover (apoptosis and necrosis), and thus, hydromineral balance was maintained (Nolan et al. 1999b).

In the present experiment, there was an absolute difference in temperature of 3–5°C between TW and RW at the beginning of and over the experimental period. This results from the fact that TW originates from groundwater, while RW is surface water. RW temperature is thus influenced by both ambient climatic factors and the many thermal discharges along its route. As these temperature differences could not be controlled during the experiment, we cannot say whether these temperature differences affected any of the parameters in the present study. There is no literature reporting the influence of such temperature differences on salmonid smolts. Gray (1990) reviewed the influence of water quality (including thermal discharges) on fish move-

ments, migration, and avoidance behavior. Natural upstream migration of sonic-tagged adult chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout in the Columbia River was not affected by thermal discharges (surface water +0–17°C), while juvenile chinook salmon migrating downstream avoided thermal discharges when plume temperature exceeded 9–11°C above ambient in laboratory experiments (Gray 1990). In relation to the cellular response to temperature change, a heat shock response in fish is induced in response to a very modest temperature increase (Iwama et al. 1998). A +4°C temperature increase resulted in the induction of stress proteins in a chinook salmon embryonic cell line (Heikkila et al. 1982). As there is a thermal gradient in the Rhine resulting from surface heating of the waters along the route and thermal discharges into the lower part of the river especially, the temperature increases experienced by the fish in RW in the present experiment are not that different from what can occur when migrating smolts pass through heavily industrialized areas.

In general, effects on hydromineral balance are a response to a variety of stressors (both real and perceived) and are brought about by the induction of an integrated stress response (Wendelaar Bonga 1997). They are, therefore, not a stressor-specific indicator or bioindicator of toxicity per se. Transient increases in gill Na^+/K^+ -ATPase activity in TW&T, RW&T, and RW without any apparent disturbance of the hydromineral balance reflect disturbance of whole-animal unidirectional ionic flow rates. In a study examining ionic flows as potential biomarkers of pollutant effects in brook trout (*Salvelinus fontinalis*), it was shown that a general response to a series of metal/pH exposure combinations was an increase in net sodium loss and elimination of the net calcium inflow that occur under normal conditions (Grippio and Dunson 1996). In freshwater-adapted rainbow trout, confinement stress for 4 or 8 h increased Na^+ and Cl^- outflow eightfold (Postlethwaite and McDonald 1995). These observations support the view that measurement of plasma electrolytes alone to assess osmoregulatory disturbance may lead to the incorrect conclusion of no effect. For such a situation, measurement of ionic flows are necessary. Increases in ion transport ATPase activities may, however, also reflect ionoregulatory disturbance (Nolan et al. 1999a, 1999b). From our experience, we conclude that the best method for assessing the effects on hydromineral balance in the absence of ion flow data is by combined plasma electrolyte and ion-transporting ATPase measurements.

The effects of the treatments on the skin and gill epithelia of the brown trout smolt provide some indication of the sublethal effects that may affect migrating brown trout in the Rhine system. Our results show that temperature shocks in the form of thermal plumes in clean water (i.e., TW) compromise the integrity of the skin and gill epithelia for a considerable period and that, in the microscope at least, these tissues appear to recover by 29 days after temperature shock. However, continuous exposure to RW alone resulted in incomplete recovery in both epithelia at 29 days. These results are in accordance with those reported for the skin of rainbow trout exposed for 24 days to RW (Iger et al. 1994c) and brown trout exposed to waste water management plant effluents (Burkhardt-Holm et al. 1997). The compromised epithelia may render the fish susceptible to ionoregulatory

disturbance and to secondary pathogenic infection during this time. Furthermore, increased occurrence and infiltration of the epithelia by leukocytes indicates effects on the immune system, probably caused by permeation of antigens across skin and gill epithelia. Exposure to chronic stress is known to lead to reduced disease resistance in fish (Pickering and Pottinger 1989; Fevolden et al. 1994). A reduced disease resistance to challenge with *Aeromonas salmonicida* related to exposure to pollutants has been reported in goldfish (*Carassius auratus*) after a 30-day exposure to 5% treated sewage (Kakuta 1997).

Significant findings in the present study are the changes in the electron-dense vesicle content of the filament cells of the brown trout smolt. These secretory vesicles have been shown to contain endogenous peroxidase activity (Iger et al. 1994a, 1994b), and their synthesis is induced in rainbow trout by administration of the primary stress hormone cortisol (Iger et al. 1995). Our data show that the numbers of these vesicles per filament cell are more than 10-fold higher in the mature brown trout smolt than in rainbow trout in the study by Iger et al. (1994a). The higher density is likely to be a species difference and may be related to the smoltification process, as the amount of vesicles illustrated in the study of Burkhardt-Holm et al. (1997) in nonsmolting brown trout epidermis is much lower than in our brown trout and similar to that in nonsmolting rainbow trout. Peroxidase has been considered to be an antimicrobial component of the nonspecific defense system of fish and has been demonstrated in the mucus and glycocalyx on the surface of the skin (Iger et al. 1994b; Brokken et al. 1998). This secreted skin peroxidase is an isoform biochemically distinct from the peroxidase of the blood. The significance of the enhanced secretion of peroxidase during stress is unknown at present (Brokken et al. 1998).

For the RW&T fish, the present data indicate that the combination of 3 h of temperature shock and RW induces the strongest effects on all parameters assessed. The significantly increased gill Na^+/K^+ -ATPase activities at 24 h and 8 days posttreatment, followed by reduced gill Na^+/K^+ -ATPase activity at 18 days, reflect events in the chloride cell population. In studies with tilapia during seawater adaptation, it has been shown that the functional, mature chloride cells degenerate during adaptation and are replaced by newly differentiated chloride cells (Wendelaar Bonga and van der Meij 1989). Reduced gill Na^+/K^+ -ATPase activity has been correlated with reduced numbers of chloride cells and increased levels of apoptotic chloride cells in the gill of seawater-adapted tilapia after confinement stress (Nolan et al. 1999b).

It is not possible to say which RW factors specifically potentiate the effects of the acute temperature shock and bring about prolonged degeneration of the epithelia and limited recovery. It may be the effects of a combination of different pollutants, even at levels that individually would have no effect. Herbicide mixtures were more toxic than individual exposures to channel catfish (*Ictalurus punctatus*) and bluegill (*Lepomis macrochirus*) (Abdelghani et al. 1997), while low pH increased heavy metal toxicity to brook trout (Grippio and Dunson 1996). Increased toxic effects of cadmium to goldfish have been shown when ammonia is present (Gargiulo et al. 1996), while greater toxicity of inorganic

contaminant mixtures was demonstrated in three endangered fish species (Buhl and Hamilton 1996). Effects of pollutants and treatments can be additive to fish, and the present study shows that the effects of temperature shock and RW are also additive.

In conclusion, a single, acute, sublethal temperature shock of $+7^\circ\text{C}$, as legally allowed at present by European legislation, induces effects within 3 h in the native brown trout smolt when delivered in TW and in present-day water from the Rhine. These effects include disrupted skin and gill epithelia that endures to 29 days posttreatment. Although 3 h of temperature elevation in dechlorinated TW resulted in good recovery by 29 days, fish temperature shocked in RW showed the least recovery. For migrating smolts in nature, who may encounter up to 30 such plumes, we speculate that these effects could lead to increased disease susceptibility and reduced hypoosmoregulatory ability in the marine environment.

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