

Intracellular proteolysis in Atlantic salmon *Salmo salar* fingerlings (0+) from different biotopes in an Arctic river (Varzuga River, White Sea Basin)

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ABSTRACT. The activity of intracellular proteolytic enzymes was studied in Atlantic salmon (*Salmo salar* L.) fingerlings (0+) after hatching from spawning nests and dispersal in the Varzuga River main stem and its Pyatka tributary (Kola Peninsula, White Sea Basin). The study focused on calcium-dependent cytosolic proteinases (μ - and m-calpains), lysosomal proteinases (cathepsins B and D) and collagenase, and determined the free/protein-bound hydroxyproline ratio, which portrays collagenolytic activity. Compared to fingerlings from the Varzuga main stem, the intracellular proteolytic enzyme activity of cysteine proteinase and collagenase was higher in fingerlings from the Pyatka tributary, where current velocities and food availability were higher. These results indicate that there is a higher rate of intracellular protein metabolism in the juveniles from this phenotypic group.

Introduction

In northern Europe, the world's largest stock of Atlantic salmon (*Salmo salar* L.) reproduces in several large rivers, including the Varzuga River. The Varzuga River is a large polar river in the White Sea Basin and has one of the largest breeding populations of Atlantic salmon. In the last century, 50,000–130,000 salmonids spawned annually in this river (Kazakov and others 1992). The hydrology of the main stem and tributaries of the Varzuga River is distinct from other rivers. There are numerous rapids and shoals located along the river, presenting spawning and nursery areas for salmonids (Veselov and Kalyuzhin 2001). Heterogeneity in the size, mass and volume of the accumulated stores of salmon eggs determine the growth rates and normal development of the fish. This heterogeneity also results in unequal abilities of the larvae to resettle in different microbiotopes, mainly regulated by rheotactic behaviour. Rheotaxis compensates for the drift of fish downstream and promotes their retention in a habitat (Veselov and others 1998). Intrapopulation heterogeneity can form in an environment and can even occur among the offspring of one pair of producers (Pavlov and others 2007). The biological significance of this heterogeneity is that it increases larval survival at the early stages of ontogenesis. Embryogenesis culminates in the Varzuga River main stem. Post-embryonic development involves the dispersal of the fry from these nests and foraging along the coast of the main stem and in tributaries. Differences in the hydrological regimes and availability of food in these habitats results in the differentiation of two phenotypic groups (Veselov and others 2005) with distinct

biochemical parameters of energy metabolism (Pavlov and others 2007, 2008).

Since the growth rate, locomotor activity and availability of energy substrates in fish depend on the rate of protein degradation in skeletal muscle, which accounts for up to 25% of all protein in muscle tissue of salmon (Mommsen 2001, 2004; Johnston and others 2011), the potential differences in intracellular proteolytic enzyme activity between salmon fingerlings are interesting. There are three known key systems of protein degradation in vertebrates: ubiquitin-proteasome, lysosomal-autophagic and calpain. The proteasome and lysosomal pathways dominate mammalian systems (Sandri 2010). In contrast, the lysosomal and calpain systems dominate in fish (Mommsen 2004; Salem and others 2005, 2006; Seiliez and others 2012, 2014).

Another essential factor for the growth and development of an organism is the proteolysis of connecting tissue in the extracellular matrix, primarily the hydrolysis of collagen, which accounts for approximately 25% of all protein in an organism. The rate of collagen metabolism can be measured by the collagenolytic activity (a matrix metalloproteinase) and by the level of free and protein-bound hydroxyproline (an amino acid unique to collagen) (Jaescke 1975; Reddy and Enwemeka 1996; Torrisen 2014).

The aim of this study was to perform a comparative analysis of the intracellular proteolysis indices in two groups of Atlantic salmon fingerlings (0+) after distribution from their spawning places to biotopes with different conditions, that is, along the coast of the Varzuga River main stem and in the Pyatka tributary.

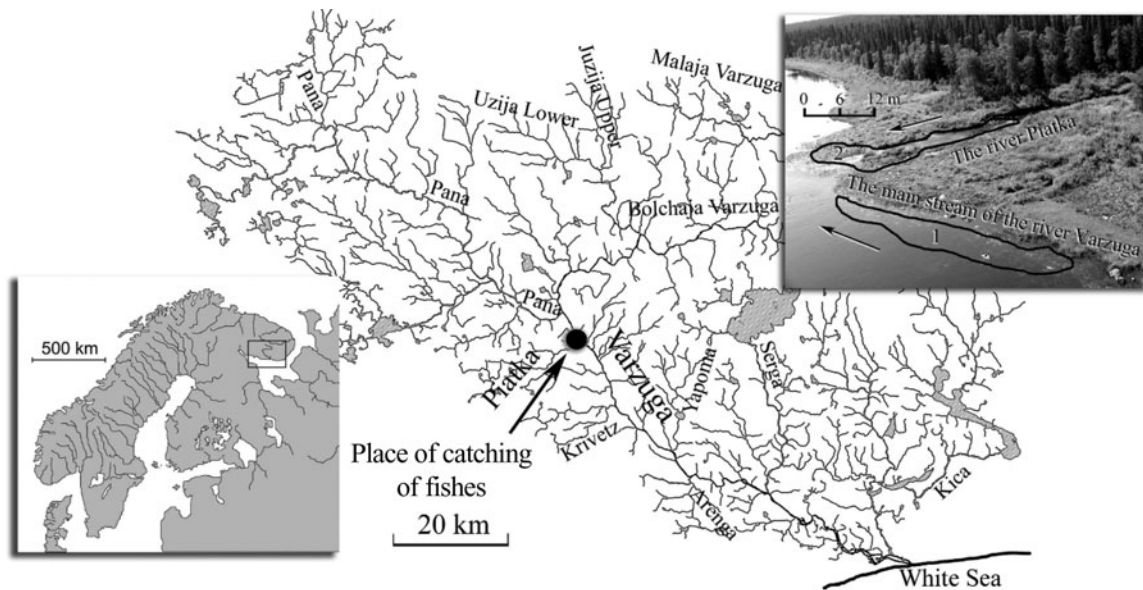


Fig. 1. The Varzuga River and sampling areas of Atlantic salmon fingerlings in the Varzuga main stem (1) and the Pyatka tributary (2).

Materials and methods

Ichthyological methods

We have previously determined (Veselov and others 2005) that Atlantic salmon annually spawned in the Varzuga River's main stem, where 8–12 nests built by several different females remained in autumn. The nests were found in sites 0.8–0.9 m deep, where current velocities at the surface were 0.8 m/sec. Current velocities were measured with a custom-constructed hydrologic whirligig (screw diameter 10 mm with a measuring range of 0.01–1.5 m/sec). No spawning took place in the downstream regions of the river. This finding, coupled with early spring electrofishing and underwater observations, suggests that after depleting the yolk sac and leaving the nests in the main stem, young salmon dispersed into different tributaries. Such tributaries are commonly small, with widths of 3–6 m and water consumption levels < 1.5 m³/s, and they flow into rapids and shoals of the main stem of the river (Veselov and Kalyuzhin 2001). To study the differences between phenotypic groups, we chose the Pyatka tributary (Fig. 1, site 2), which flows into a spawning site in the main stem (Fig. 1, site 1). By the time the fingerlings occupied these biotopes, they had gained scales and juvenile colouring, signifying the end of the larval stage and the beginning of the fry stage.

Atlantic salmon fingerlings were collected with an electrofishing device designed for research purposes (Norway, Fa-2) (Zippin 1956). Samples were collected twice; first, on 15 June 2007, soon after the fry had reached the coastline and the tributary mouth, and then on 30 July 2007. To eliminate electroshock stress, the collected fish were held for 24 h in cages located in the main stem of the river. Length and weight measurements were taken for each fish. The sampled biological material (120 specimens from each biotope) was then frozen *in situ* in liquid

nitrogen and delivered to the laboratory for biochemical analysis.

Biochemical methods

Intracellular proteolytic enzyme and collagenolytic activity were determined individually ($n = 25$ –30) in whole organisms. Calpain activity was measured in composite samples from 8–10 specimens (5 g aliquots, $n = 5$), as it involved chromatographic separation of tissue proteins.

Quantitative analysis of soluble protein levels in tissues (mg/g tissue) was performed based on the method from Bradford (1976), with bovine serum albumin as the standard. Standard assays for proteolytic activity in animal tissues, including ectothermic tissues, enabled the estimation of maximal proteolytic (or peptidase) activity under standardised conditions.

Lysosomal proteinase activity

Cysteine proteinase cathepsin B and aspartic cathepsin D are the main proteinases of lysosomes (Bohley 1987). After homogenising with an Ultra-Turrax homogeniser, the samples were mixed 1:10 with a 0.25 M sucrose solution with 0.01% Triton X-100 (Merck) (1,200 rpm, 60 sec) and centrifuged (30 min, 10,000 g; Allegra 64R Beckman Coulter). Cathepsin B (EC 3.4.22.1) activity in the supernatant fraction was determined by the digestion of 0.065 M N-benzoyl-L-arginine ethyl ester HCl solution in 0.1 M acetate buffer (pH 5.0) (Matsuda and Misaka 1974), and cathepsin D (EC 3.4.23.5) activity was determined using 1% bovine haemoglobin hydrolysis in 0.1 M acetate buffer (pH 3.6) following a modified Anson method (Barrett and Heath 1977). The activity of cathepsin B and D (activity units) was expressed as units of change in optical absorption (E_{525} and E_{280} , respectively) per 1 mg protein per 1 h incubation at 37°C.

Activity of calcium-dependent proteinases

The samples were homogenised with an Ultra-Turrax homogeniser in 20 mM Tris-HCl buffer (pH 7.5) with 80 mM KCl, 5 mM Na-EDTA and 20 mM dithiothreitol in a 1:3 ratio (w/v) and centrifuged (4°C, 20,000 rpm, 15 min); then, the supernatant (soluble fraction) was applied to a 2.5 x 95 cm column with Sephacryl S-300 (Pharmacia) equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 4 mM EDTA and 5 mM mercaptoethanol. Chromatographic separation of the samples enabled the isolation of a calpain-specific inhibitor, calpastatin, and the fractionation of individual molecular forms of the enzyme. Separation was made at 4°C using standard LKB-Pharmacia devices. Fractions were recorded on a Uvicord II absorptiometer at 280 nm. The activity of calpains (EC 3.4.22.17), calcium-induced activity, was determined in 4 ml eluent fractions using a standard technique of casein hydrolysis (Murachi and others 1981). The reaction mixture for calpain activity determinations was a 2.5 ml total volume containing 0.4% casein, 5 mM dithiothreitol, 50 mM imidazole-HCl buffer (pH 7.5) and enzyme solution. Experimental samples were incubated in the presence of CaCl₂ solutions (at μ M and mM concentrations) and calcium was added to control samples after incubation. After a 30 min incubation at 30°C, the reaction was terminated by adding an equal volume of 10% trichloroacetic acid. The concentration of acid-soluble hydrolysis products was determined by spectrophotometry (E₂₈₀). Calpain activity units were defined as the change in optical density (E₂₈₀) over a 30 min incubation and taken as 1 IU. After chromatographic separation of the salmon fingerling homogenates, fish calpains with μ M and mM sensitivity to calcium were detected, which can be identified as homologues of mammalian μ -calpain and m-calpain (Salem and others 2006; Bondareva and others 2006).

Three distinct protein fractions exhibiting calcium-dependent proteolytic activity and differing in calcium sensitivity were identified in salmon fingerling homogenates (Nemova and others 2014). Fish calpains with μ M and mM calcium sensitivity can be identified as homologues of mammalian μ -calpain and m-calpain, respectively, and the low-molecular component is a catalytically active subunit of the named calpains (Sorimachi and Suzuki 2001; Bondareva and others 2006).

Determination of collagenase (EC 3.4.24.3) activity

The method for determining enzyme activity was based on the incubation of enzyme-containing biological fluid with collagen followed by a hydroxyproline-based determination of the protein degradation products (Jaescke 1975; Reddy and Enwemeka 1996). Units of enzyme activity were defined as the amount in 1 g wet weight resulting in the release of 1 μ M hydroxyproline.

Determination of free and protein-bound hydroxyproline

Quantitative determinations of free and protein-bound hydroxyproline were based on conventional techniques

(Jaescke 1975; Reddy and Enwemeka 1996). The material was fixed with ethanol in a 1:10 ratio. The fixed material was centrifuged at 4,000 g for 15 min. The supernatant fraction was removed and the pellet was rinsed with 5 ml of 80% ethanol and centrifuged using the same settings. The resulting extract was dried in a water bath and the dry residue containing free hydroxyproline was dissolved in 1 ml of 10% isopropanol. To isolate protein-bound hydroxyproline, the dry residues of fish tissues were hydrolysed in vacuum sealed ampoules with 6N HCl for 24 h at 105°C after the extraction of free amino acids. The hydrolysate was filtered and the filtrate was dried in a water bath. The HCl was removed by repeatedly (5–6 times) dissolving the dry residue in water and evaporating, after which the dry residue was dissolved in 1 ml of 10% isopropanol. Hydroxyproline levels (μ M/g tissue) were calculated using a calibration curve based on the standard dilutions of hydroxyproline.

Statistical treatment of the results

To compare groups, a non-parametric Mann–Whitney *U* test was used for parameters without normal distribution, such as proteinase activity and hydroxyproline content. The data were analysed to determine whether they exhibited a normal distribution using the StatGraphics package and Excel.

Results

Fingerling dispersal and formation of phenotypic heterogeneity

Fingerlings (0+) dispersed from the nests to the Pyatka tributary and the littoral zone of the Varzuga River main stem in June at high water when temperatures reached \geq 12°C. This process lasted 6–8 days. Fingerlings moved from the nursery area to the main stem surface and were then carried downstream by the current.

The hydrological regime in the dispersal areas changed daily as the water level in the Varzuga River lifted by 9–10 cm per day, reducing flow velocities and causing multiple streams to form between large exposed boulders. A daily 5–6 cm decline in water level also occurred in the tributary, affecting the power of the current. As a result, young salmon that moved earlier mostly reached the tributary, whereas those dispersing several days later settled along the main stem littoral biotopes. Fish larvae have an innate rheotactic behavioural response to the current (Pavlov 1979; Veselov and others 1998) and move actively against the direction of the current along the littoral zone of the main stem to tributaries. Over the course of two weeks after resettlement, the water level and current flow rate significantly decreased in both the main stem and in the tributary. As a result, the fingerlings in both the tributary and the main stem were sedentary and no migration of larvae between the main stem of the river and the tributary was observed.

As previous studies have shown, the temperature of the water was higher in the Pyatka tributary than in the main

Table 1. Characteristics of salmon fingerlings and the biotopes in the Varzuga main stem and the Pyatka tributary.

Characteristic	Varzuga main stem	Pyatka tributary
Body length (AC), cm		
15 June 2007	2.22 ± 0.04	2.28 ± 0.04
30 July 2007	4.00 ± 0.20	4.30 ± 0.20
Body weight, g		
15 June 2007	0.26 ± 0.04	0.27 ± 0.05
30 July 2007	0.43 ± 0.03*	0.63 ± 0.03*
Fry density, ind./m ²	1–3	4–5
Depth, m	0.15–0.45	0.10–0.30
Flow velocity at water surface, m/sec	0.4–0.7	0.7–1.0
Food capacity ^a , weight of invertebrates from 1 m ² of bottom	Low: < 2 g/m ²	High: > 10 g/m ²
Type of ground fractions of the riverbed	Gravel, insufficient shelter	Bouldery gravel, unlimited shelter
Size fractions of riverbed ^b , %		
Sand (< 0.25 cm)	5%	3%
Gravel (0.26–0.5 cm)	6%	3%
Fine pebble (0.6–1.5 cm)	9%	5%
Medium pebble (1.6–2.5 cm)	18%	7%
Coarse pebble (2.6–5.0 cm)	33%	13%
Small boulder (5.1–10.0 cm)	14%	39%
Medium boulder (11–25 cm)	11%	17%
Large boulder (26–50 cm)	3%	12%
Very large boulder (> 51 cm)	1%	1%
Water temperature, °C	17.2	18.7

main stem* Differences reliable at $P \leq 0.05$.

^aAfter Shustov 1983.

^bAfter Veselov and Kalyuzhin 2001.

stem during the day, whereas at night, the opposite was observed. In the tributary, the temperature fluctuated by 5–6°C during the day whereas in the main stem, temperature fluctuations were around 2–3°C during the day in June.

It has been shown that for young salmon (0+) the foraging conditions are more favourable in the small tributaries of the Varzuga River compared with the main stem (Baryshev and others 2005). Some of the characteristics of the biotopes inhabited by the salmon fingerlings are presented in Table 1. Fingerlings from the Pyatka tributary inhabited areas with higher flow velocity (0.6–0.9 m/sec versus 0.4–0.7 m/sec) and made more darts for food (23 ± 3 in 15 min versus 14 ± 5 in 15 min; calculated by AEV during underwater observations) during the day in sunny weather compared to juveniles in the main stem. Fish density in the tributary was twofold higher (Table 1) due to favourable foraging conditions in the biotope. Larger areas of exposed ground contribute to greater isolation of fingerlings from each other, resulting in decreased competition. Higher current flow rates in the tributaries mean they are richer sources of food. Daily temperature oscillations and good foraging conditions can advance the growth of juveniles in the tributaries compared with those in the main stem (Konstantinov and others 2007).

The first sampling effort was made soon after the emergence of fry from spawning nests (15 June 2007). In these samples, the length and weight measurements of fish were similar between the two biotopes (Table 1). In

July, the fingerlings collected from the Pyatka tributary had higher body weight (1.5-fold) compared with fish from the main stem.

Activity of proteolytic enzymes

The results of the analysis of selected proteolytic enzyme activity and soluble protein content in salmon fingerlings from the different biotopes of the Varzuga River are presented in Table 2.

The soluble protein content in young salmon was not significantly different between the Pyatka tributary and the Varzuga main stem. The activity of most of all intracellular proteolytic enzymes studied was significantly higher in the fingerlings from the Pyatka tributary compared with the fish from the main stem. However, lysosomal aspartic proteinase cathepsin D activity was not significantly different between the biotopes.

The fingerlings from the Pyatka tributary had approximately 1.3-fold higher total activity of calcium-dependent proteinases, mainly due to a higher (5.7-fold) activity of m-calpain, which depends on mM concentrations of calcium. The activity of lysosomal cysteine proteinase cathepsin B in fingerlings from the Pyatka tributary was also significantly higher (1.3-fold) than in fish from the main stem.

The fingerlings from the studied biotopes also differed significantly in the level of collagenolytic activity. Collagenase activity in fish from the Pyatka tributary was

Table 2. Activity of proteolytic enzymes and protein content in salmon fingerlings from the Varzuga main stem and the Pyatka tributary ($n = 25-30$).

Variable	Biotope	
	Varzuga main stem	Pyatka tributary
Protein concentration (mg/g tissue)	43 ± 0.6	48 ± 0.8
Total calpain activity (activity units)	0.22 ± 0.01	0.47 ± 0.02*
μ-calpain activity (activity units)	0.15 ± 0.01	0.07 ± 0.01*
m-calpain activity (activity units)	0.07 ± 0.01	0.40 ± 0.03*
Cathepsin D activity (activity units)	5.60 ± 0.4	5.35 ± 0.4
Cathepsin B activity (activity units)	42.10 ± 2.0	55.50 ± 2.5*
Free hydroxyproline (μM/g tissue)	80.90 ± 6.2	118.10 ± 7.8*
Protein-bound hydroxyproline (μM/g tissue)	1260.50 ± 90.5	246.20 ± 20.7*
Collagenolytic activity (activity units)	0.08 ± 0.01	0.16 ± 0.01*

* $P \leq 0.05$.

1.3-fold higher compared with fish from the Varzuga main stem. In the fingerlings from the main stem, levels of protein-bound hydroxyproline were fivefold lower and levels of free hydroxyproline were twofold higher than in the fish from the Pyatka tributary.

Discussion

Salmonids have a complex life cycle including different developmental stages maintained by a complex system of adaptations. Significant morphological and physiological changes during ontogenesis, as prerequisites of future smoltification, occur when fish inhabit river sites. It was shown in this study that salmon fingerlings (at 0+ age) resettle and inhabit different biotopes of the main channel of the river and its tributaries (including the Pyatka tributary) after hatching. Differences in the hydrologic and foraging conditions between the main stem and the tributary lead to the formation of two phenotypic groups and determine the further differentiation of juveniles. The foundation for these processes is significant changes in cell metabolism. Intensive proteolysis at an early stage of post-embryonic development, when young fish are adapting to the external environment, is very important for active growth and further development in salmon (Overturf and Gaylord 2009; Valente and others 2013; Lysenko and others 2015). Differences in the activity of intracellular proteolytic enzymes in salmon fingerlings from biotopes with distinct ecological conditions support the above statement. The elevated activity of cysteine proteinases m-calpain and cathepsin B, which have a central role in regulating limited proteolysis (Attaix and others 1999), indicates a high rate of protein metabolism in the fingerlings from the Pyatka tributary. Moreover, protein metabolism has two components: protein synthesis and degradation. Limited proteolysis plays an important role in the post-translational processing of synthesised proteins. The correlation between m-calpain and cathepsin B may be due to calcium homeostasis. For example, Mizote and others (1999) demonstrated that cathepsin B in *Xenopus* eggs promotes intracellular Ca^{2+} release from endoplasmic reticulum calcium stores, increasing the

concentration of cytoplasmic Ca^{2+} , which is considered to be an activator of calpains.

Increased activity of calpains may be associated with higher levels of ionised cytoplasmic calcium in the cytoplasm of cells, as well as with a higher content of membrane cholesterol and certain phospholipids, which could act as direct regulators of calpain activity in the cell (Tomba and others 2001; Lysenko and Nemova 2012; Murzina and others 2014). Increased activity of calcium-dependent proteolytic activity, mainly of m-calpain (5.7-fold), in fingerlings from the Pyatka tributary was correlated with a higher locomotor activity of juveniles based on calcium homeostasis in muscles. Higher locomotor activity of an organism is known to be closely associated with muscle tissue calcium homeostasis (Cottin and others 1994; Seebacher and others 2012).

Studies of the biochemical parameters of collagen metabolism in young salmon (0+) showed that individuals from the Pyatka tributary had higher collagenase activity and free hydroxyproline content (with a simultaneous reduction in protein-bound hydroxyproline) compared with fish from the Varzuga main stem, indicating a relatively higher rate of collagen metabolism in the former group. The functions of collagen are diverse as it supports the specific structure of organs and tissues during the development and life cycle of an organism. Collagen, like other proteins, is subjected to catabolic and anabolic processes. Catabolised collagen provides energy and catabolites, and thus a continuous metabolism. We assume that our findings, higher collagenase activity and changes in the ratio of free hydroxyproline to protein-bound hydroxyproline found in juveniles from the Pyatka tributary, reflects the role of these proteins in the metabolism of connective tissue during the growth and development of young salmon. Furthermore, in addition to specific collagenases, collagenolysis is thought to involve lysosomal proteinases (Eherington and Evans 1977), which is supported by our data showing elevated collagenolytic and lysosomal proteinase cathepsin B activity in fingerlings from the Pyatka tributary. Thus, we have demonstrated a relationship between the functional activity of the selected enzymes and intracellular proteolysis.

The environmental conditions in the biotopes we studied had less effect on the activity of lysosomal aspartic proteinase cathepsin D in the fingerlings. It may be that anabolism dominates over catabolism during the studied period of early post-embryogenesis in young fish. Bohley (1987) proposed that the principal function of cathepsin D is complete proteolysis of protein molecules into dipeptides and amino acids. It is possible that changes in the activity of the lysosomal endopeptidase would be found in the next stages of development of juveniles, when degradation processes prevail over synthesis.

Conclusion

The ecological conditions along the main stem coast and in a tributary of the Arctic Varzuga River contribute to the differentiation of Atlantic salmon fingerlings and the formation of two phenotypic groups. We assume that more favourable hydrologic conditions in the Pyatka tributary biotope (higher locomotor activity of the fish, frequent and fast darts for food, prolonged periods of feeding during the polar day, and greater availability of food) are the main factors contributing to the intensive growth and development of the salmon juveniles. This process is maintained by higher rates of metabolism (catabolism and anabolism of structural components) including proteolysis. The fingerlings inhabiting the Pyatka tributary demonstrated a relatively higher activity of intracellular proteolytic enzymes (cysteine-dependent and collagenolytic), indicating a higher rate of intracellular protein metabolism. It could be expected that this differentiation in fingerlings continues in further differences between the juveniles, ultimately resulting in different smoltification ages.

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Supplementary material

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