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SHORT COMMUNICATION

Ammonia first? The transition from cutaneous to branchial ammonia excretion in developing rainbow trout is not altered by exposure to chronically high NaCl

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ABSTRACT

Larval rainbow trout (*Oncorhynchus mykiss*) were reared from hatch under control ($[Na^+]$=0.60 mmol l^{-1}) or high NaCl ($[Na^+]$=60 mmol l^{-1}) conditions to elucidate the driving force for the ontogeny of branchial Na^+/NH_4^+ exchange, one of the earliest gill functions. We hypothesized that if Na^+ uptake is the driving force, then in high NaCl there would be a delay in the skin-to-gill shift in ammonia excretion (J_{amm}) and/or an elevation in whole-body total ammonia (T_{amm}). In both groups, however, the skin-to-gill shift for J_{amm}, determined using divided chambers, occurred at the same time (13 days post-hatch; dph) and whole-body T_{amm} was unchanged. Moreover, high NaCl larvae displayed elevated whole-body $[Na^+]$ relative to controls by 18 dph, suggesting that maintaining branchial J_{amm} occurs at the expense of Na^+ balance. Overall, these results support the 'ammonia hypothesis', which posits that ammonia excretion, probably as Na^+/NH_4^+ exchange, is the primary function of the early fish gill.

KEY WORDS: Ammonia excretion (J_{amm}), Ammonia hypothesis, Gill development, Ionoregulatory hypothesis, *Oncorhynchus mykiss*, Na^+ uptake, Na^+/K^+-ATPase

INTRODUCTION

In most fish species, the gill is the primary site for physiological exchanges of respiratory gases, ions, acid–base equivalents and nitrogenous wastes with the environment. However, following hatch, larval fish possess only a rudimentary gill, and therefore the skin of these fish performs the majority of the physiological functions typical of the gill of adult fish (see Brauner and Rombough, 2012 for review). Several studies have investigated the ontogenetic pressures underlying gill development in larval fish. The original idea of August Krogh (1941) was the 'oxygen hypothesis' – i.e. that gill development was driven by the increasing need for O_2 uptake. However, the most recent evidence supports the 'ionoregulatory hypothesis', which posits that ionoregulatory demand, in one form or another, is the driving force for branchial ontogeny (see Brauner and Rombough, 2012 for review). To date, two studies have provided direct support for this hypothesis using a divided chamber approach to demonstrate that in developing larval rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792), Na^+ uptake shifts from the skin to the gills prior to oxygen uptake (Fu et al., 2010; Zimmer et al., 2014). However, what remains unclear is which aspect of ionoregulation can be considered the earliest function of the developing gill.

Brauner and Rombough (2012) suggest that it is unlikely that branchial ion uptake in early life stages serves the sole purpose of mineral nutrient acquisition but, rather, that ion uptake probably occurs as coupled exchanges, such as those involved in acid–base regulation and/or N-waste excretion. In typical freshwater fish, branchial Na^+ uptake is believed to occur in part via Na^+/NH_4^+ exchange, which is coordinated by an exchange complex of a number of different transporters (see Wright and Wood, 2009 for review). In larval rainbow trout, the skin-to-gill shift for Na^+ uptake occurs in synchrony with that of ammonia excretion (J_{amm}) and branchial Na^+ uptake is closely correlated with branchial J_{amm} throughout development (Zimmer et al., 2014). However, there is no such relationship between skin Na^+ uptake and skin J_{amm} (Zimmer et al., 2014). Thus, the development of branchial Na^+ uptake occurs as a linked Na^+/NH_4^+ exchange process. In addition, the ontogeny of branchial Na^+/NH_4^+ exchange is coincident with increased branchial gene expression and enzyme activity of the transporters involved in Na^+/NH_4^+ exchange (Zimmer et al., 2014).

But is the driving force for the development of branchial Na^+/NH_4^+ exchange the need for Na^+ uptake or the need for ammonia excretion? To address this question, larval rainbow trout were raised from hatch up to 18 days post-hatch (dph) under control conditions (approximately 0.6 mmol l^{-1} NaCl) or in water containing a high concentration of Na^+ (approximately 60 mmol l^{-1} NaCl), comparable to the concentration observed in post-hatch larvae (Brauner and Wood, 2002). Over developmental time, whole-body $[Na^+]$ increases (Brauner and Wood, 2002), probably associated with increasing extracellular fluid (ECF) volume. We hypothesized that by providing larvae with greatly increased ambient $[Na^+]$, there would be a reduction in ionoregulatory demand, potentially delaying the skin-to-gill shift for Na^+ uptake. From this hypothesis, we predicted that if Na^+ uptake is driving the development of branchial Na^+/NH_4^+ exchange, there would be a delayed skin-to-gill shift in J_{amm} in the high NaCl group relative to control fish. In contrast, if ammonia excretion is the driving force, the skin-to-gill shift in J_{amm} would be unaffected by the reduced ionoregulatory demand imposed by high ambient NaCl. We assessed this idea using a divided chamber approach to quantify J_{amm} in the gills versus the skin at 3, 6, 9, 12 and 18 dph. Whole-body $[Na^+]$ and Na^+/K^+-ATPase activity in the gill, yolk sac skin and body skin were also assessed over the 18 day period as indicators of ionoregulatory status/capacity in both groups of fish. We further predicted that Na^+/K^+-ATPase activity, which increases over larval development at least in the gills (e.g. Fu et al., 2010; Zimmer et al., 2014), probably to support increasing whole-body $[Na^+]$, would be down-regulated in the gill, body skin and/or yolk sac skin in high ambient NaCl, reflecting a reduction in ionoregulatory demand.

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Received 13 January 2015; Accepted 10 March 2015

The Journal of Experimental Biology (2015) 218, 1467-1470 doi:10.1242/jeb.119362

RESULTS AND DISCUSSION

Very clearly, there was no delay in the overall skin-to-gill shift in J_{amm} in the high ambient NaCl treatment relative to the control group (Fig. 1A,B). The 50% transition point occurred at 13.0±1.0 days in controls (Fig. 1A) and 13.5±0.9 days in the high NaCl group (Fig. 1B), values that were not significantly different. In addition, whole-body T_{amm} was unchanged by rearing larvae in high NaCl (P=0.144) and embryo mass in the two treatment groups was essentially the same at all time points (Table 1), indicating that developmental rate was also not affected. Overall, this result suggests that the requirement for increased ammonia excretion over development is the key driver in the temporal shift of ionoregulatory function to the gills from the skin. The need for an effective mechanism to excrete metabolic ammonia in developing larvae may be particularly important given the eventual loss of ureagenicity, which is critical for ammonia detoxification in the embryonic stage (Wright et al., 1995). Moreover, the transition points reported here were very similar to those reported previously for skin-to-gill shifts for J_{amm} (15.0±0.7 dph: Zimmer et al., 2014) and Na^+ uptake (15–16 dph: Fu et al., 2010; and 14.9±1.2 dph: Zimmer et al., 2014) in a range of different freshwater Na^+ concentrations (0.06–2.3 mmol l^{-1}) but at similar temperatures (10–12°C). These observations lend further support to the 'ammonia hypothesis' – that ammonia excretion is the primary function of the developing gill. It appears that the timing of the skin-to-gill transition for J_{amm} is 'pre-programmed' into the developmental blueprint so as to ensure adequate excretion of this toxic N-waste, such that it cannot be altered by Na^+ availability.

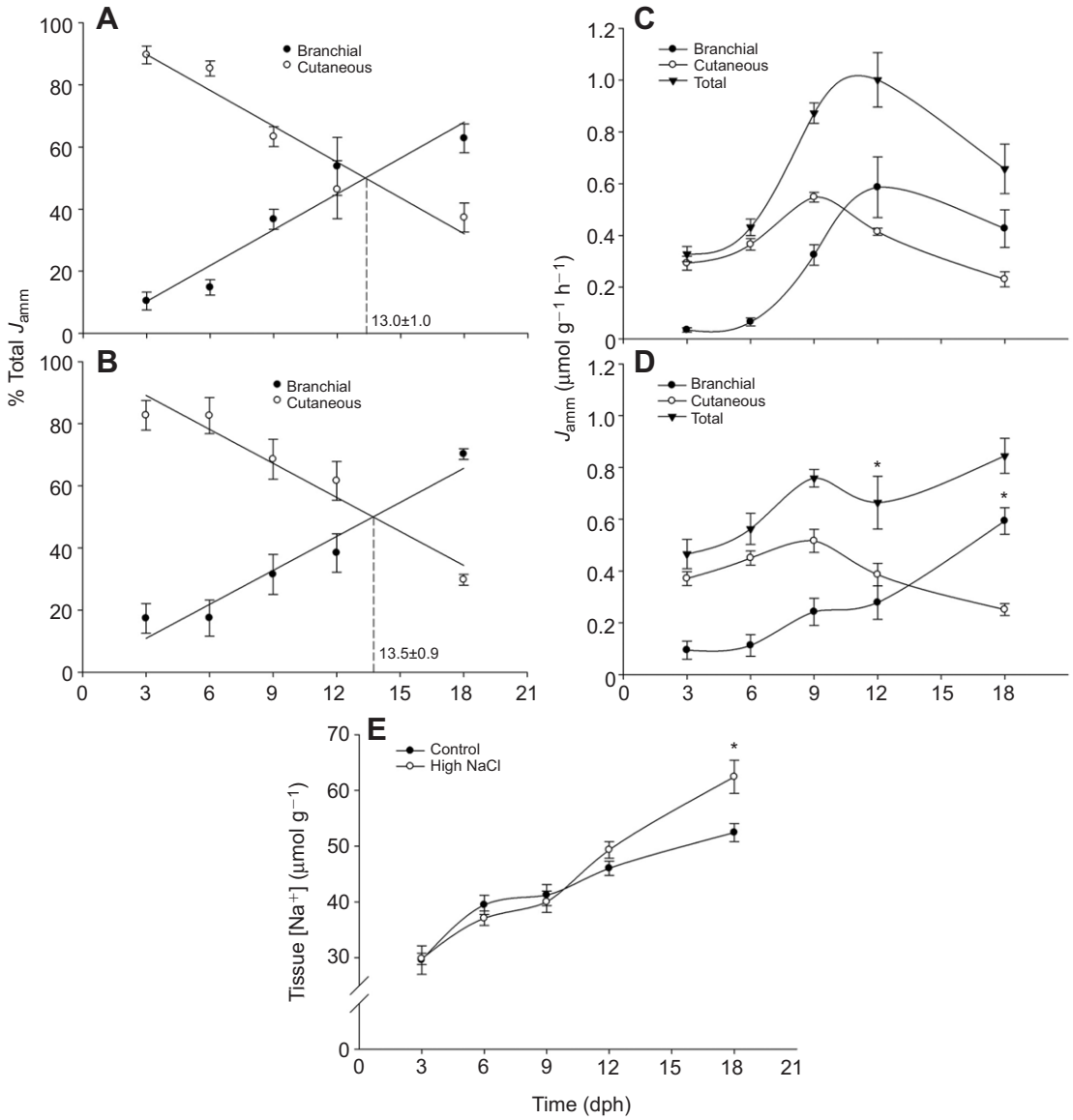

Fig. 1. The influence of chronic high NaCl exposure on the temporal pattern of J_{amm} partitioning between gills and skin, and on whole-body [Na^+] in developing trout larvae. (A,B) The percentage of total cutaneous and branchial ammonia excretion (J_{amm}) in control larvae (A) and high NaCl larvae (B) over 18 days post-hatch (dph). Dashed lines in A and B represent the skin-to-gill shifting points for J_{amm}. (C,D) Absolute branchial, cutaneous and total J_{amm} in control (C) and high NaCl (D) larvae over 18 dph. (E) Tissue [Na^+] in control and high NaCl larvae over 18 dph. In D, asterisks represent statistically significant differences (P<0.05) in mean branchial, cutaneous or total mean J_{amm} between control and high NaCl larvae at a given time point. In E, asterisks represent statistically significant differences between mean tissue [Na^+] in control and high NaCl larvae at given time points. All comparisons were made using a two-way ANOVA, with time and treatment as factors, followed by a Holm–Sidak *post hoc* test (N=6–8).

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Table 1. Larval mass, whole-body T_{amm} and ammonia turnover time of larvae raised under control and high NaCl conditions over 18 days post-hatch

	Mass (g)		Whole-body T_{amm} (μmol g^{-1})		Ammonia turnover time (h)	
Time (dph)	Control	High NaCl	Control	High NaCl	Control	High NaCl
3	0.076±0.002	0.074±0.004	1.70±0.18	1.98±0.26	5.42±0.44	4.63±0.52
6	0.080±0.003	0.080±0.005	2.37±0.44	2.74±0.33	5.68±0.48	4.81±0.38
9	0.085±0.003	0.089±0.005	3.82±0.36	4.30±0.20	4.42±0.20	5.71±0.25
12	0.098±0.003	0.097±0.005	3.16±0.63	3.28±0.22	3.45±0.57	5.72±1.10*
18	0.098±0.005	0.093±0.004	1.99±0.39	2.58±0.56	3.44±0.63	3.15±0.22

T_{amm}, total ammonia concentration; dph, days post-hatch.
Asterisks denote statistically significant differences (P<0.05) between control and high NaCl groups as determined by a two-way ANOVA using time and treatment as factors, followed by a Holm–Sidak *post hoc* test (N=4–8).

From 3 to 18 dph, the overall increase in total J_{amm} in the two groups was relatively similar (100% and 82% increases in control and high NaCl groups, respectively), although some temporal differences were observed (Fig. 1C,D). At 12 dph, there was a significant 35% depression in total J_{amm} in the high NaCl treatment relative to the controls, and this occurred entirely at the gills (Fig. 1C,D). In addition, ammonia turnover time was also significantly increased (Table 1). This relative decrease in branchial J_{amm} in the high NaCl group occurred just as the gills were starting to come into use, which might suggest that branchial Na^+/NH_4^+ exchange was transiently delayed. Subsequently, at 18 dph, J_{amm} was restored to control levels, occurring entirely via an increase in branchial J_{amm} (Fig. 1C,D).

Up to the transition point, the two groups exhibited almost identical temporal profiles of whole-body [Na^+], with approximately 50% increases from day 3 to day 12 (Fig. 1E). Clearly, prior to the transition point, whole-body [Na^+] is carefully regulated. Very probably, the rate of active Na^+ uptake was greatly reduced in the high NaCl larvae prior to the skin-to-gill shift at 13 dph, though it is also possible that the rate of Na^+ excretion was greatly increased in these fish. By 18 dph, whole-body [Na^+] increased 20% in the high NaCl group relative to controls (Fig. 1E), and this occurred at the same time that branchial J_{amm} was also increased significantly relative to the control group (Fig. 1C,D), further supporting the ammonia hypothesis. Here, we are comparing instantaneous J_{amm} and cumulative whole-body [Na^+], but the same conclusion can be drawn by comparing cumulative whole-body T_{amm} and [Na^+]. Note that whole-body [Na^+] was increased significantly by 18 dph in high NaCl while whole-body T_{amm} was unchanged throughout the exposure, again suggesting that the primary function of the early fish gill is to ensure ammonia excretion, even at the expense of Na^+ balance.

We had initially predicted that in the face of more favourable gradients for Na^+ uptake in high NaCl, there would be an attenuation of the increase in branchial Na^+/K^+-ATPase activity normally seen over development (Fu et al., 2010; Zimmer et al., 2014; Table 2). However, this was not the case, and Na^+/K^+-ATPase activities were not responsive to high NaCl treatment (Table 2). Overall, it appeared that the high NaCl group had a tendency, though not statistically significant (P=0.075), to increase branchial Na^+/K^+-ATPase activity, a phenomenon seen in adult trout acclimated to seawater (Richards et al., 2003) or fed a high NaCl diet (Perry et al., 2006).

In summary, our main finding is a lack of plasticity in the ontogeny of branchial ionoregulation in developing rainbow trout larvae, thereby ensuring that requirements for ammonia excretion are met. These results support those of Fu et al. (2010), showing a similar lack of plasticity in the ontogeny of Na^+ uptake. Our new ammonia hypothesis refines the ionoregulatory hypothesis, suggesting that the need to excrete ammonia, probably via Na^+/NH_4^+ exchange, is the primary function of the developing gill and the main ontogenetic pressure for gill development.

MATERIALS AND METHODS

Experimental design

Rainbow trout (*O. mykiss*) were purchased in the eyed-up stage from Rainbow Springs Hatchery (Thamesford, ON, Canada) and held in flow-through Hamilton dechlorinated tap water (moderately hard: [Na^+]=0.6 mequiv l^{-1}, [Cl^-]=0.8 mequiv l^{-1}, [Ca^{2+}]=1.8 mequiv l^{-1}, [Mg^{2+}]=0.3 mequiv l^{-1}, [K^+]=0.05 mequiv l^{-1}; titration alkalinity 2.1 mequiv l^{-1}; pH ∼8.0; hardness ∼140 mg l^{-1} as $CaCO_3$ equivalents, 12°C). Fish hatched approximately 1 week after purchase and were then randomly separated into two different treatments: control ([Na^+]=0.70±0.00 mmol l^{-1}) and high NaCl ([Na^+]=58.29±2.03 mmol l^{-1}), the latter made by adding NaCl to dechlorinated tap water. In initial trials, higher levels of ambient [NaCl] (up to 140 mmol l^{-1}) were tested, but caused increased mortality after a few days. Therefore, a nominal concentration of 60 mmol l^{-1}, the highest level not causing mortality, was selected. Both treatments were conducted at 12°C using static, aerated systems in which the water was completely renewed on a daily basis. At 3, 6, 9, 12 and 18 dph, J_{amm} was measured in a divided chamber system using randomly selected larvae from both groups. Also, at the above experimental times, separate larvae from both treatments were randomly selected for the measurement of whole-body tissue T_{amm} and [Na^+]. At 6, 12 and 18 dph, other randomly selected larvae from each treatment were removed

Table 2. Na^+/K^+-ATPase activities in the gill, yolk sac epithelium and body epithelium of larvae raised under control and high NaCl conditions over 18 days post-hatch

	Gill (μmol ADP mg^{-1} protein h^{-1})		Yolk sac epithelium (μmol ADP mg^{-1} protein h^{-1})		Body epithelium (μmol ADP mg^{-1} protein h^{-1})	
Time (dph)	Control	High NaCl	Control	High NaCl	Control	High NaCl
6	1.15±0.16	1.07±0.21	0.89±0.14	0.55±0.12	1.38±0.75	1.11±0.24
12	1.70±0.27	2.38±0.32	0.96±0.16	1.27±0.16	1.00±0.28	1.39±0.35
18	2.02±0.36	3.00±0.61	1.64±0.79	1.95±0.67	1.04±0.38	2.04±0.84

dph, days post-hatch.
Data were analysed by a two-way ANOVA using time and treatment as factors, followed by a Holm–Sidak *post hoc* test (N=4–8); no statistically significant differences were found between control and high NaCl groups.

for the determination of Na^+/K^+-ATPase activity in gill, yolk sac epithelium and body epithelium. Because of the high Na^+ concentration (60 mmol l^{-1}), it was not feasible to measure Na^+ uptake as it would require an excessive quantity of radioisotope to obtain the necessary specific activity.

Divided chamber ammonia fluxes

J_{amm} in both groups was measured in a divided chamber system closely following the protocol of Zimmer et al. (2014). The only changes were that fish from the respective treatment groups were loaded into divided chambers containing either control water with 0.05 mg l^{-1} neutralized MS-222 or high NaCl water with the same concentration of neutralized MS-222. In addition, [^{22}Na] (Perkin Elmer, Waltham, MA, USA) was added to one chamber to a final concentration of 0.001 µCi ml^{-1} for the sole purpose of ensuring that there were no leaks in the dividing dam. All fluxes were 1 h in duration. Fish recovered from MS-222-induced anaesthesia within 5 min following flux, and isotope leak from the ^{22}[Na]-loaded chamber to the unloaded chamber was less than 10%. Collected water samples from fluxes were stored at −20°C until subsequent analysis of T_{amm} using methods described previously (Zimmer et al., 2014). J_{amm} (µmol g^{-1} h^{-1}) in anterior and posterior chambers was calculated using the following equation:

$$J_{amm} = \frac{[(T_{amm,f} - T_{amm,i}) \times V]}{(M \times t)}, \quad (1)$$

where $T_{amm,f}$ and $T_{amm,i}$ are final and initial concentrations of ammonia (µmol l^{-1}), respectively, of water samples from the anterior or posterior chamber, V is volume of the respective chamber (l), corrected for sample removal, M is larva mass (g) and t is flux duration (h). From these values, branchial and cutaneous J_{amm} were determined by correcting for J_{amm} across the cutaneous surface (skin of the head) in the anterior chamber following calculations and cutaneous surface area estimates used previously (Zimmer et al., 2014).

The skin-to-gill shifting point was then calculated by plotting percentage total cutaneous and percentage total branchial J_{amm} means as x-values against time in dph as y-values. x=0 was then set to 50% by subtracting 50% from all plotted means and a linear regression was used to determine the y-intercept (x=50%), which corresponded to the time in dph at which 50% of total J_{amm} occurred cutaneously or branchially, i.e. the skin-to-gill shifting point.

Tissue T_{amm} and [Na^+]

At the times outlined above, larvae from each experimental group were removed, anaesthetized with an overdose of neutralized MS-222, rinsed with distilled water, flash-frozen in liquid N_2 and stored at −80°C. These samples were later ground to a fine powder under liquid N_2 in a chilled mortar and pestle, and 150 µl of 8% perchloric acid was added to approximately 50 mg of powdered sample. These samples were kept on ice for 5 min for deproteinization before 5 min centrifugation at 8000 rpm at 4°C; 10 µl of the resulting supernatant was stored at 4°C for later [Na^+] analysis. The remainder of the supernatant was neutralized using 3 mol l^{-1} KOH, flash-frozen and kept at −80°C until later T_{amm} analysis.

[Na^+] in tissue homogenates was measured by atomic absorption spectrophotometry (Varian Model 1275, Mulgrave, VIC, Australia). T_{amm} in deproteinized and neutralized homogenates was measured using a commercial enzymatic assay (Raichem Ammonia Assay, Cliniqa, San Marcos, CA, USA). Ammonia turnover time (h) was calculated using the following equation:

$$\text{Turnover time} = \frac{\text{Mean whole-body } T_{amm}}{\text{Total } J_{amm}}. \quad (2)$$

Na^+/K^+-ATPase activity

At 6, 12 and 18 dph, larvae from both treatment groups were killed with an overdose of neutralized MS-222. Whole gill baskets, yolk sac skin and body skin were then dissected from the fish under a stereomicroscope, flash-frozen and stored at −80°C. Tissues were later homogenized in an EGTA-deoxycholate buffer and Na^+/K^+-ATPase activity was determined as previously described (Zimmer et al., 2014) based on a protocol modified from McCormick (1993). Activity was normalized to protein content determined with a commercial protein assay (Bio-Rad, Hercules, CA, USA) and a bovine serum albumin standard (Sigma, St Louis, MO, USA).

Statistics

All data are presented as means±s.e.m. (N=sample size). All statistical analyses were performed using a 2-way ANOVA with treatment and time as factors, followed by a Holm–Sidak *post hoc* test using SigmaStat v. 3.5 (Systat Software, Inc.). In Fig. 1 and Table 1, significant differences between treatments (P<0.05) are denoted by asterisks. The skin-to-gill shifting points (together with corresponding s.e.m.), represented by dashed lines in Fig. 1A,B, were determined using SigmaPlot v.10.0 (Systat Software, Inc.).

Acknowledgements

We thank Dr Colin Brauner for inspiring the initial concept of this study and for helpful comments on the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

A.M.Z. executed the experiments, analysed and processed samples, contributed to the design of the experiments and drafted the manuscript. C.M.W. contributed to experimental design and interpretation of results, and revised the manuscript.

Funding

This research was funded by a Natural Sciences and Engineering Research Council (NSERC) Discovery Grant (RGPIN473-12) to C.M.W. who was also supported by the Canada Research Chairs Program. A.M.Z. is supported by an NSERC Canada Graduate Scholarship.

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