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Effects of desalted Noto deep ocean water on plasma glucose and cortisol in freshwater goldfish and salivary amylase activity in humans

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Key words: deep ocean water, desalination, kynurenine, stress markers, physiological regulation.

Abstract

Deep Ocean Water (DOW) has attracted attention for applications in aquaculture and health-related research. We previously reported that Noto DOW, pumped from a depth of 320 m in Toyama Bay, Japan, was associated with reduced physiological stress-related markers in marine organisms, partly through the action of the tryptophan metabolite kynurenine. However, it remains unclear whether desalted Noto DOW retains biologically active components and exerts similar physiological effects in freshwater organisms and humans. In this study, we investigated the effects of desalted Noto DOW on physiological stress-related markers in freshwater goldfish and humans. Chemical analysis confirmed the presence of kynurenine in desalted Noto DOW. Goldfish maintained in desalted Noto DOW exhibited significantly lower plasma glucose and plasma cortisol levels than those kept in tap water. In human participants, ingestion of desalted Noto DOW significantly reduced salivary amylase activity, whereas no significant change was observed following consumption of commercially available natural water. These findings indicate that desalted Noto DOW retains detectable low-molecular-weight organic compounds after desalination and is associated with

changes in physiological stress-related markers in freshwater goldfish and salivary amylase activity in humans. However, the bioactive component(s) responsible for these effects and the underlying mechanisms remain to be determined.

Introduction

Deep ocean water (DOW) is seawater collected from depths of ≥200 m below the ocean surface and is characterized by low temperature, high cleanliness, and enrichment in inorganic nutrients.[1,2] These properties have led to growing interest in the use of DOW in aquaculture, food production, and health-related research.[3,4] Human studies have shown that intake of desalinated DOW modulates physiological responses to physical exertion, as evidenced by accelerated recovery of physical performance, reduced elevations of muscle damage–related biomarkers,[5] and improved cerebrovascular and inflammatory responses following exertion.[6] In addition, sustained consumption of DOW has been reported to improve metabolic profiles in humans, including reductions in serum total cholesterol and low-density lipoprotein cholesterol levels.[7] Consistent improvements in lipid metabolism have also been observed in experimental mammals.[8] These effects have been attributed primarily to the mineral composition of DOW, which is enriched in physiologically active ions such as magnesium (Mg^{2+}) and calcium (Ca^{2+}).[5-8]

Noto DOW is pumped from seawater at a depth of 320 m in Toyama Bay, Japan, and is characterized by high cleanliness and enrichment in inorganic minerals.[9,10] We have previously demonstrated that rearing marine organisms in Noto DOW confers beneficial physiological effects, including suppression of body weight loss in squid (*Todarodes pacificus*)[10] and reductions in plasma cortisol levels, a widely used indicator of physiological stress,[11] in Japanese flounder (*Paralichthys olivaceus*) and nibbler fish (*Girella punctata*) under high-density rearing conditions.[9-12] Notably, kynurenine, a bioactive metabolite of tryptophan[13] present in Noto DOW, was shown to suppress high-density induced elevations in plasma cortisol levels in Japanese flounder,[9] indicating that this low molecular weight organic compound contributes to the physiological effects of Noto DOW in addition to the inorganic minerals typically present in DOW.[14,15]

However, it remains unclear whether biologically active low-molecular-weight compounds, including kynurenine, are retained after desalination of Noto DOW and whether desalted Noto DOW influences physiological responses in freshwater organisms and humans. Moreover, despite accumulating evidence for the biological effects of Noto DOW in marine organisms,[9,10,12] its physiological effects in freshwater organisms and humans have not been systematically examined.

Therefore, we investigated whether desalted Noto DOW retains kynurenine and related low-molecular-weight metabolites, whether it influences plasma glucose and plasma cortisol levels, two physiological stress-related markers,[11,16] in freshwater goldfish, and whether it alters salivary amylase activity, a commonly used noninvasive physiological stress marker,[17,18] in human participants.

Materials and Methods

Preparation of the desalted Noto DOW

The geographical location of the Aquas Noto facility in Ishikawa Prefecture, Japan, together with the intake point from which Noto DOW used in the present study was collected, is shown in Figure 1. The preparation procedure for desalted Noto DOW is outlined in Figure 2. Pumped Noto DOW was initially passed through a #40 mesh and subsequently filtered using bag filters (BP420-25 and BP420-5; Kyoshin Techno Co., Ltd., Osaka, Japan) with pore sizes of 25 μm and 5 μm, respectively. The filtered water was stored in a 10 m^3 tank and further passed through a 3-μm filter prior to desalination using a reverse osmosis membrane system (AWT-SW4O4OH; Nitto Denko Corp., Osaka, Japan). The desalted water was not remineralized after reverse osmosis desalination, and neither pH nor osmolarity was adjusted or standardized before use. It was then sterilized by ultraviolet irradiation and stored in a 5 m^3 food-grade polyethylene tank managed under the standard sanitary cleaning and quality-control procedures used for the commercial production of drinking water. All processing and transport were carried out under ambient temperature conditions. The resulting sterilized desalted Noto DOW was used for all experiments in the present study.

Analysis of indole compounds in desalted Noto DOW

Desalted Noto DOW (3 L) was passed through C18 extraction disks (3M 2215 C18 Empore Extraction Disks; GL Sciences, Tokyo, Japan) under reduced pressure using an aspirator (AS ONE Corporation, Osaka, Japan). The retained compounds were eluted with 10 mL ethanol followed by 30 mL benzene. The eluate was dried under a stream of nitrogen gas and reconstituted in 200 μL of Milli-Q water (Veolia Japan GK, Tokyo, Japan). Immediately prior to analysis, samples were stirred for 5 min, centrifuged, and passed through a 0.22-μm filter. The resulting solutions were subjected to liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis.
Indole compounds in desalted Noto DOW were simultaneously analyzed using a previously described LC–MS/MS method.[19] The analytes included kynurenine, kynurenic acid, and tryptophan. Aliquots (10 μL) were injected into a high-performance liquid chromatography system (LC30-AD; Shimadzu, Kyoto, Japan) equipped with a C18 column (TSKgel ODS-100V, 2.0 × 150 mm, 3 μm;

Tosoh, Tokyo, Japan). The mobile phase consisted of 10 mmol/L ammonium acetate in 0.05% (v/v) acetic acid with increasing concentrations of methanol. A linear gradient from 5% to 50% methanol was applied over 20 min, followed by 100% methanol for 10 min, at a flow rate of 0.3 mL/min. The autosampler and column oven were maintained at 4°C and 25°C, respectively.
Detection was performed using a triple quadrupole mass spectrometer (LCMS-8050; Shimadzu, Kyoto, Japan) operated in multiple reaction monitoring mode. The monitored transitions (m/z) were 208.65→191.90 for kynurenine, 189.95→143.95 for kynurenic acid, and 204.80→188.05 for tryptophan. The limits of sensitivity at a signal-to-noise ratio of 2:1 were 11.43 pg for kynurenine, 0.52 pg for kynurenic acid, and 3.86 pg for tryptophan. The intra-assay coefficients of variation were 1.51%, 1.50%, and 2.75%, respectively.

Animals

Thirty-eight male goldfish (19.8 ± 0.49 g) were obtained from Higashikawa Fish Farm (Yamatokoriyama, Nara, Japan). The fish were acclimated in tap water at 25°C for one week prior to experimental use.

Measurement of plasma cortisol levels in goldfish

Plasma cortisol levels were quantified by LC–MS/MS. Plasma samples (100 μL) were mixed with methanol (400 μL), vortexed for 5 min, and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant (100 μL) was filtered through a 0.22-μm membrane filter (Ultrafree-MC-GV; Merck Millipore, Burlington, MA, USA) by centrifugation at 10,000 rpm for 2 min. Aliquots (10 μL) were injected into the same LC–MS/MS system and chromatographic conditions described above for indole compound analysis. The monitored transition (m/z) was 363.0→121.1 for cortisol. The limit of sensitivity (signal-to-noise ratio = 2:1) was 0.14 pg, and the intra-assay coefficient of variation was 1.31%.

Measurement of plasma glucose concentration in goldfish

Each goldfish was accommodated individually in an 8-L glass tank containing either desalted Noto DOW or tap water for 3 days at 18 ± 1°C. Fish were anesthetized with 0.03% ethyl 3-aminobenzoate methanesulfonic acid salt, neutralized with sodium bicarbonate, and blood samples were collected from the caudal vessels using a syringe and transferred to heparinized tubes. Plasma was obtained by centrifugation at 5,200 × g for 5 min, and glucose concentrations were measured using a commercial glucose assay kit (L-Type Glu 2; FUJIFILM Wako Pure Chemical Corp., Osaka, Japan).

Measurement of osteoblastic and osteoclastic activities of goldfish scales

After the maintenance period, scales were collected from goldfish anesthetized with ethyl 3-aminobenzoate methanesulfonic acid salt (MS-222; Sigma-Aldrich, St. Louis, MO, USA). Individual scales were placed into separate wells of 96-well microplates, and 100 μL of either Alkaline Phosphatase (ALP) or Tartrate-Resistant Acid Phosphatase (TRAP) buffer was added to each well. The plates were immediately frozen at −80°C and subsequently stored at −20°C until analysis.

ALP and TRAP activities were measured as previously described.[20] After thawing, ALP activity was determined by adding 100 μL of alkaline buffer (100 mM Tris-HCl, pH 9.5, containing 1 mM $MgCl_2$) supplemented with 20 mM *p*-nitrophenyl phosphate to each well. Plates were incubated at 20°C for 20 min at gentle shaking. The reaction was terminated by adding 50 μL of 3 N NaOH, and 150 μL of the reaction mixture was transferred to a new plate. Absorbance was measured at 405 nm using a microplate reader (SH-1300, Corona Electric Co., Ltd., Ibaraki prefecture, Japan) and converted to the amount of p-nitrophenol produced using a standard curve.

TRAP activity was measured under identical conditions, except that an acid buffer (0.1 M sodium acetate buffer, pH 5.3, containing 20 mM tartrate) was used. After enzymatic assays, the surface area of each scale was measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA), and ALP and TRAP activities were normalized to scale surface area (cm^2).

Measurement of plasma calcitonin levels

Plasma calcitonin levels were measured using a competitive Enzyme-Linked Immunosorbent Assay (ELISA) based on a previously described method,[21] with minor modifications.[22] Amino group–coated plates (ELISA Plate Amino; Sumitomo Bakelite Co., Ltd., Tokyo, Japan) were used, allowing the preincubation period to be shortened from 3 days to 1 day.[22]

The specificity of the rabbit anti-salmon calcitonin serum (No. 626; Cosmo Bio Co., Ltd., Tokyo, Japan) was evaluated using bovine parathyroid hormone peptide (1–34) and human calcitonin gene-related peptide, as previously described.[21] Owing to the high sequence homology (91%) between goldfish and salmon calcitonin,[23] this antibody has been widely used to assess plasma calcitonin levels in goldfish.[24]

Subjects

Twenty-three healthy adults (10 women and 13 men) aged >20 years were recruited for this study. The effects of desalted Noto DOW and commercially available natural water were evaluated in each

subject, with a minimum interval of 3 h between assessments. The order of water intake (desalted Noto DOW or commercially available natural water, specifically Happy Belly Natural Water Gifu/Yoro) was randomized for each subject.

Saliva collection and measurement of salivary amylase activity

Saliva samples were collected 2 h after breakfast or lunch. Subjects rinsed their mouths and rested in a seated position for 5 min before sample collection. Saliva was collected immediately before and 5 min after consumption of 100 mL of desalted Noto DOW or commercially available natural water (Happy Belly Natural Water Gifu/Yoro), both administered at room temperature, following an additional 5-min seated rest period. A minimum interval of 3 h was maintained between assessments, and the order of water intake was randomized for each subject. Salivary α-amylase activity was measured using a salivary amylase monitor (Nipro Corp., Osaka, Japan) according to the manufacturer's instructions.

Statistical analyses

Homogeneity of variance was assessed using an F-test, and plasma glucose and plasma cortisol levels in goldfish were compared between the control and experimental groups using Student's *t*-test. Changes in salivary amylase activity before and after consumption of desalted Noto DOW or commercially available natural water were evaluated using paired *t*-tests or Wilcoxon signed-rank tests, as appropriate. A p value of <0.05 was considered statistically significant.

Results

Presence of kynurenine in desalted Noto DOW

Because tryptophan, kynurenine, and kynurenic acid are metabolites of the kynurenine pathway that have been implicated in physiological stress-related processes,[13,25,26] we quantified their concentrations in desalted Noto DOW using LC–MS/MS. Measurable concentrations of tryptophan, kynurenine, and kynurenic acid remained detectable in desalted Noto DOW after desalination (Table 1).

Effects of desalted Noto DOW on plasma glucose and cortisol levels in goldfish

Goldfish maintained in desalted Noto DOW for 3 days showed significantly lower plasma glucose levels than those reared in tap water (Figure 3A). Plasma cortisol levels were also significantly lower in fish reared in desalted Noto DOW than in those reared in tap water (Figure 3B).

Effects of desalted Noto DOW on bone cell activities of the scales and plasma calcitonin levels in goldfish

In Japanese flounder, we previously demonstrated that Noto DOW reduced plasma cortisol levels through enhanced calcitonin production by scale osteoblasts, as functionally inferred from reduced osteoclastic activity.[9] Based on this, we evaluated ALP and TRAP activities, which correspond to osteoblastic and osteoclastic activities, respectively, in goldfish scales as well as plasma calcitonin levels. No significant differences in any of these parameters were observed between goldfish reared in desalted Noto DOW and those reared in tap water (Figure 4).

Effects of desalted Noto DOW on salivary amylase activity in humans

At the group level, salivary amylase activity was significantly lower 5 min after ingestion of desalted Noto DOW than before ingestion, despite inter-individual variability (Figure 5A). In contrast, no significant change was observed following consumption of the commercially available natural water (Figure 5B). Moreover, the change in salivary amylase activity differed significantly between the desalted Noto DOW and natural water groups (Figure 5C).

Discussion

To determine whether the stress marker–reducing effect of Noto DOW previously reported in marine fish[9,10,12] is also observed in freshwater species, we examined plasma glucose and plasma cortisol in goldfish reared in desalted Noto DOW. In the present study, desalted Noto DOW decreased plasma glucose and plasma cortisol levels without affecting osteoblastic or osteoclastic activities in the scales or plasma calcitonin levels. This response differs from our previous findings in marine fish, in which kynurenine present in Noto DOW was associated with reduced osteoclastic activity and activation of a scale-based calcitonin signaling pathway involved in stress regulation.[9] The lack of changes in scale bone cell activities or plasma calcitonin levels in goldfish suggests that the reductions in plasma glucose and cortisol are unlikely to be mediated primarily through calcitonin-related signaling in scale tissue. These findings indicate that the mechanisms underlying stress marker–reducing effects of desalted Noto DOW may differ between marine and freshwater fish, potentially reflecting differences in endocrine responsiveness, mineral homeostasis, and osmoregulatory physiology.[27]

Kynurenine is a bioactive tryptophan metabolite that functions not only as a metabolic intermediate but also as a neuroendocrine signaling molecule involved in the modulation of stress-related physiological responses.[28,29] Because kynurenine remained detectable after filtration and desalination, it remains one of several candidate compounds that may contribute to the observed

decreases in plasma glucose and plasma cortisol levels in freshwater fish. Kynurenic acid and tryptophan were also retained after processing and may likewise contribute to the physiological effects of desalted Noto DOW. These findings suggest that retained low-molecular-weight organic metabolites may contribute to the regulation of stress-related physiological markers, although the underlying mechanisms remain unclear.

The Noto DOW used in the present study was subjected to mechanical filtration followed by reverse osmosis desalination before use. These processing procedures are expected to alter the physicochemical composition of DOW by removing or reducing certain dissolved and particulate constituents. Because the relative effects of mechanical filtration and reverse osmosis desalination were not examined separately, it remains unclear how each processing step influenced the composition of the final desalted DOW. Because freshwater fish are highly responsive to changes in environmental ionic conditions, which influence osmoregulatory and endocrine physiology,[27] processing-related changes in water composition should also be considered when interpreting the present findings. Therefore, although retained low-molecular-weight organic metabolites remain plausible candidates contributing to the observed physiological responses, the possible influence of changes in inorganic constituents or particulate components cannot be excluded. Further studies are needed to clarify the relative contributions of these factors to the biological effects of desalted Noto DOW.

Extending our analysis beyond aquatic organisms, we further demonstrated that ingestion of desalted Noto DOW significantly reduced salivary amylase activity in human participants. Salivary amylase is widely used as a noninvasive indicator of sympathetic nervous system activation, and its reduction is generally interpreted as reflecting attenuation of physiological stress-related responses.[17,18] No comparable change was observed following consumption of commercially available natural water, indicating that the observed effect is not attributable to water intake per se but rather to components present in desalted Noto DOW.

Because salivary amylase activity was measured only 5 min after ingestion, it is unlikely that the observed response resulted from *de novo* enzyme synthesis. Rather, if desalted Noto DOW directly influenced salivary amylase activity, a rapid mechanism involving modulation of enzyme secretion and/or autonomic nervous system activity would be more plausible. It should also be noted that the concentration of kynurenine detected in desalted Noto DOW was substantially lower than endogenous concentrations reported in human plasma (approximately 1.8 μM).[30] Therefore, the present human study does not provide sufficient evidence to attribute the observed reduction in salivary amylase activity to a systemic effect of kynurenine. Although desalted Noto DOW altered

salivary amylase activity, the responsible bioactive component(s) and the underlying mechanism remain to be determined.

An additional limitation of the human study is that the commercially available natural water used as the control was not specifically matched to desalted Noto DOW with respect to physicochemical properties, including pH and mineral composition. Therefore, the possibility cannot be excluded that differences in physicochemical properties contributed to the observed changes in salivary amylase activity. Future studies using control waters more closely matched in physicochemical properties will be necessary to clarify the factor(s) responsible for the observed response.

DOW has traditionally attracted attention for its inorganic constituents, particularly magnesium and other minerals with metabolic effects.[7,31,32] Consistent with this view, previous human and animal studies have reported improvements in lipid metabolism, exercise recovery, and inflammatory responses following DOW or deep-sea water intake.[5-8] The present study extends these observations by demonstrating that biologically active low-molecular-weight compounds remain detectable after desalination of Noto DOW. However, because water processing alters the overall composition of DOW, the relative contributions of retained organic compounds and processing-related changes in water composition to the observed physiological responses remain unclear. Further studies will be required to identify the bioactive component(s) responsible for the observed physiological responses and to clarify their mechanisms of action.

Individual housing has been reported to alter physiological stress responses in some fish species[33] and therefore may have influenced the measured parameters in the present study. However, because all fish were maintained under identical housing conditions, isolation stress alone is unlikely to account for the differences observed between the experimental groups. Nevertheless, the possibility that interactions between housing conditions and water composition contributed to the observed physiological responses cannot be completely excluded. Further studies using alternative housing conditions will be necessary to clarify this issue.

Another limitation of the present study is the relatively small sample size in the goldfish experiment (n = 9 per group). Although significant differences in plasma glucose and plasma cortisol levels were detected, the limited sample size may have reduced the statistical power of the analysis and increased the influence of individual variability or outliers on the estimated effect size. Therefore, further studies with larger sample sizes are needed to confirm the robustness and reproducibility of these findings and to provide more precise estimates of the physiological effects of desalted Noto DOW.

Conclusions

The present study demonstrated that desalted Noto DOW retained detectable low-molecular-weight organic compounds after desalination. Goldfish reared in desalted Noto DOW exhibited significantly lower plasma glucose and plasma cortisol levels than those reared in tap water, whereas ingestion of desalted Noto DOW significantly reduced salivary amylase activity in human participants. No significant changes were detected in scale bone cell activities or plasma calcitonin levels in goldfish, suggesting that the physiological responses observed in freshwater fish differ from those previously reported in marine species. Together, these findings indicate that desalted Noto DOW is associated with changes in physiological stress-related markers in freshwater goldfish and salivary amylase activity in humans. Further studies are required to identify the bioactive components responsible for these effects and to clarify the underlying mechanisms.

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Table 1. Concentrations of indole compounds in desalted Noto deep ocean water

Compound	**Concentration (pg/L)**
Kynurenine	21.86
Kynurenic acid	96.45
Tryptophan	237.91

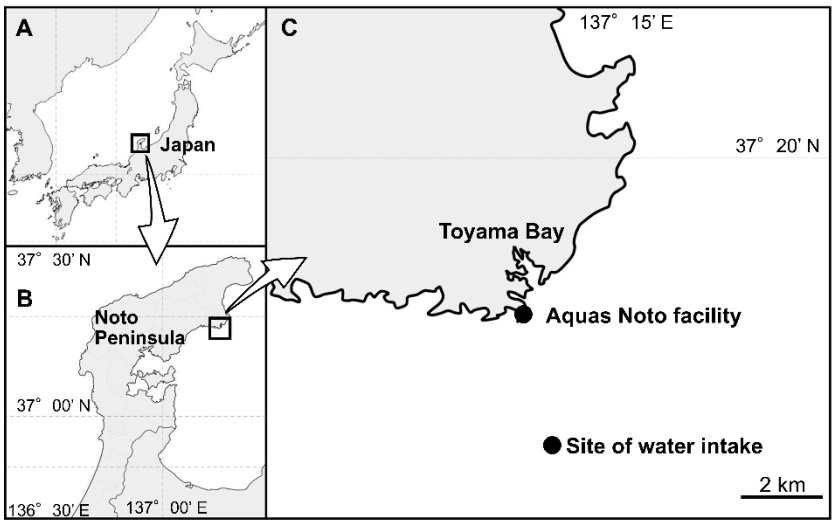

Figure 1. Geographical location of the Aquas Noto facility in Ishikawa Prefecture, Japan, and the corresponding intake a point for Noto deep ocean water used in the present study.

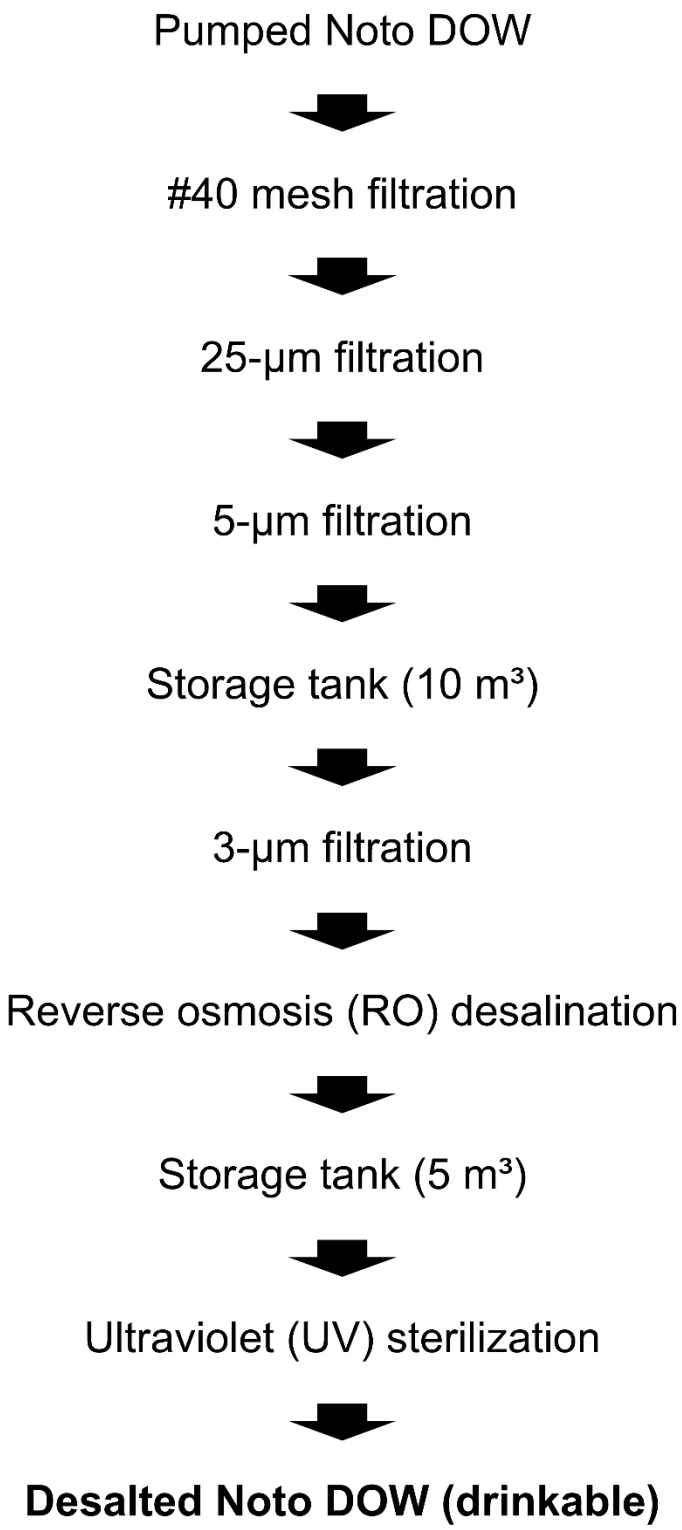

Figure 2. Schematic illustration of the preparation procedure for desalted Noto deep ocean water (DOW) used in the present study.

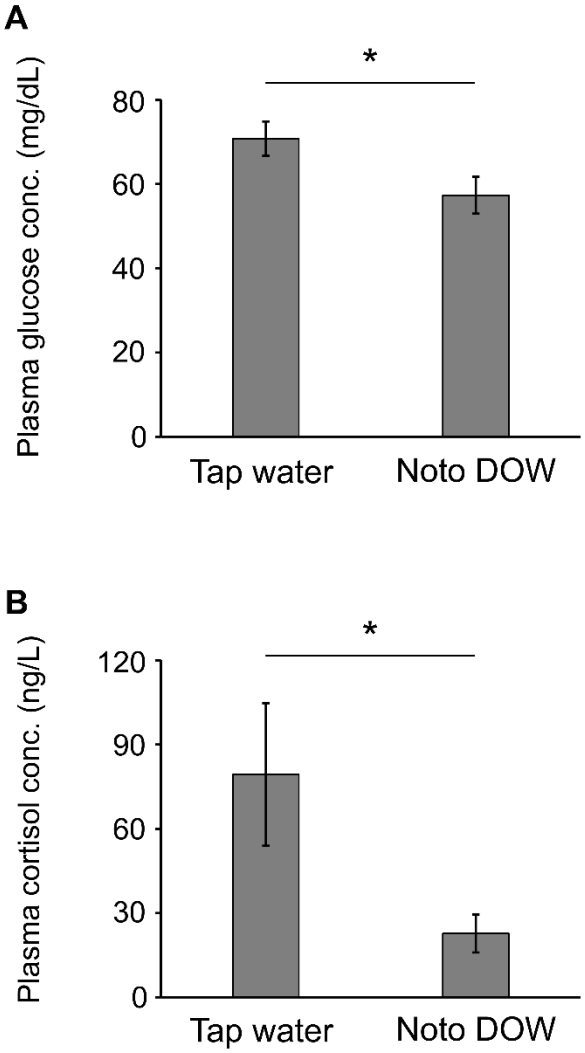

Figure 3. (A) Plasma glucose levels in goldfish maintained in tap water (n = 9 per group) or desalted Noto Deep Ocean Water (DOW). * $p < 0.05$. (B) Plasma cortisol levels in goldfish maintained in tap water (n = 9) or desalted Noto deep ocean water (DOW, n = 11). * $p < 0.05$.

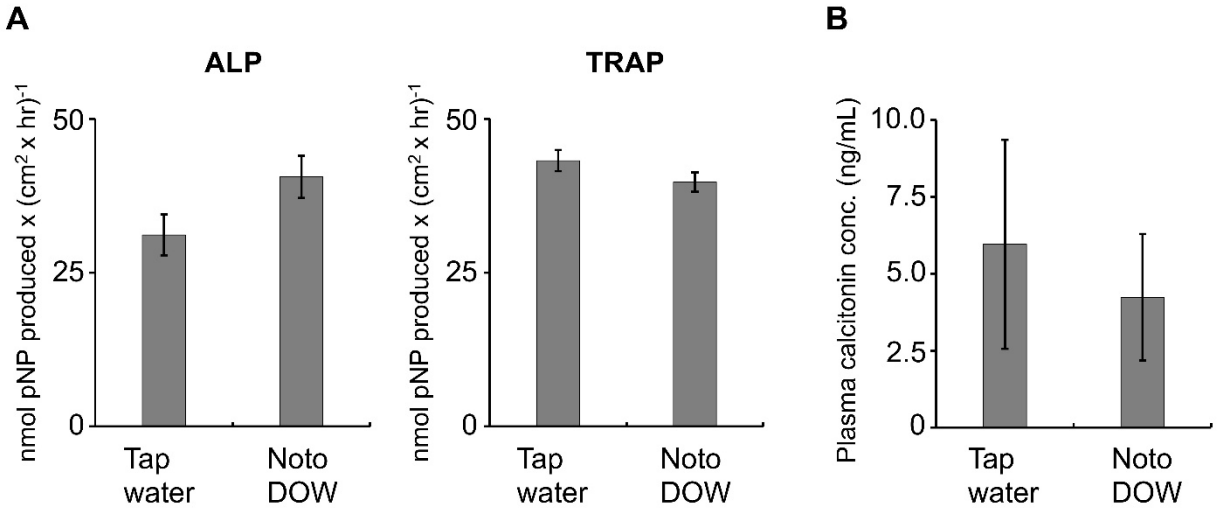

Figure 4. Alkaline phosphatase (ALP; left) and tartrate-resistant acid phosphatase (TRAP; right) activities in the scales (A), and plasma calcitonin levels (B), of goldfish maintained in tap water or desalted Noto deep ocean water (DOW) for 3 days (n = 9 per group).

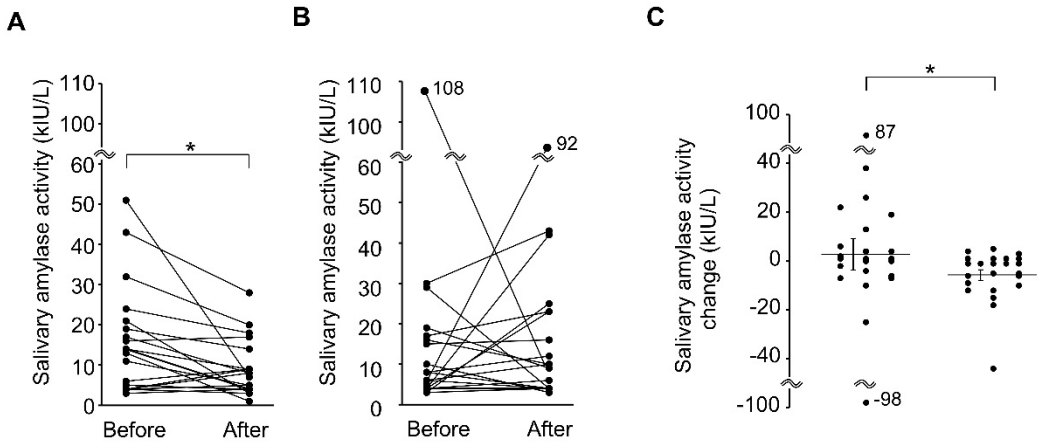

Figure 5. Salivary amylase activity levels in study participants before and after drinking desalted Noto deep ocean water (DOW) or commercially available natural water. (A) Salivary amylase activity measured before and after ingestion of desalted Noto DOW (100 mL) in all subjects (n = 23). * $p < 0.05$. (B) Salivary amylase activity measured before and after ingestion of commercially available natural water (100 mL) in the same subjects (n = 23). (C) Comparison of changes in salivary amylase activity after ingestion of commercially available natural water (left) and desalted Noto DOW (right) * $p < 0.05$.

Contributions: Tasuku Hirose, Kenji Toyota, and Nobuo Suzuki conceptualized the study, designed the experiments, performed the majority of the experiments, and drafted the manuscript. Yusuke Maruyama, Masato Honda, Kohei Kuroda, Kaori Taira, Shouzo Ogiso, Harumi Takino, Yusuke Oyadomari, and Haruyasu Kato performed additional experiments, analyzed the data, and contributed to manuscript editing. Jun Hirayama, Yoshiaki Tabuchi, and Atsuhiko Hattori analyzed the data and contributed to writing and editing the manuscript. All authors read and approved the final manuscript.

Conflict of interest: the authors declare that they have no competing interests.

Ethics approval: all procedures involving human participants were reviewed and approved by the Clinical Studies Ethics Committee of Kanazawa University (approval no. 2020-03; approved on December 9, 2020). Written informed consent was obtained from all participants prior to participation. Participation was voluntary, and all data were anonymized to ensure confidentiality. All animal experiments were conducted in accordance with the ethical guidelines of Kanazawa University and were approved by the Animal Welfare Committee of Kanazawa University (approval no. AP24-010; approved on April 11, 2024). Experiments were performed in compliance with the ARRIVE guidelines (version 2.0).[34] Pain and distress were minimized through the appropriate use of anesthesia during invasive procedures, and anesthetic protocols were carefully managed to reduce animal suffering.

Data availability statement: all other data supporting the findings of this study are available within the manuscript file.

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