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Ocean Acidification's Potential Effects on Keratin Protein in Cetacean Baleen and Other Integumentary Tissue

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ABSTRACT

Marine uptake of atmospheric CO2 from increased anthropogenic carbon emissions is leading to ocean acidification, which poses a grave threat to marine life. The potential risk of acidified seawater to cetaceans (whales, dolphins, and porpoises) and other marine mammals has received little attention, but deserves close scrutiny due to their long lifespan. Cetaceans also lack the protective fur coat which protects typical mammals, and the feeding of mysticete whales depends on a filter made of a unique tissue: baleen. Like hair and other integumentary products, baleen is made of keratin, a fibrous structural protein. We submerged baleen and skin samples from bowhead whales (Balaena mysticetus) and North Atlantic right whales (Eubalaena glacialis) for 12 weeks in seawater of varying pH representing current and projected acidification. When tested for mechanical strength via loading in compression and tension, the acid-exposed specimens were slightly but not statistically different in weakness (as measured by deformation for a given stress or the force needed to fracture the sample). Other samples exposed to low pH were examined via nuclear magnetic resonance testing to search for the presence of amino acids expected if the keratin protein deteriorated due to acid exposure; even at extreme acidity these amino acids were not found, suggesting keratin is strongly resistant to acid-induced breakdown. Finally, whale skin samples exposed to acidified seawater and examined microscopically did not demonstrate notable changes in appearance, texture, or resistance to stretching. Ocean acidification can possibly harm keratin-based cetacean tissues, but no acid-related effects were conclusively demonstrated by these tests.

***Keywords:** ocean acidification, pH, keratin, whale, baleen, skin, epithelium*

INTRODUCTION

Ocean acidification is a well-documented phenomenon. This chemical change is due to increased atmospheric CO_2 from anthropogenic and natural carbon emissions [1]. As CO_2 and water interact via carbonic acid, bicarbonate and hydrogen ions form, lowering pH [2]. Over the past 100 years ocean pH has dropped about 0.1 unit, from 8.2 to 8.1, but since the pH scale is logarithmic, this represents a nearly 30% increase in H^+ over the past 200 years [3]. Current projections estimate a further decrease of 0.3 to 0.5 pH units by the end of this century, representing an additional doubling or tripling of protons (H^+ ions) [4].

Numerous field and laboratory studies have examined the effects of ocean acidification (OA) on marine life. These range from diminished ability of mollusks to form calcareous shells to neurological problems in fish [5]. To date the potential consequences of OA on mammals have received little attention [6]. Our study was conceived and conducted to investigate the prospective effects of current and future declines in pH on the integumentary structures of mammals, and in particular the keratin protein [7-10] of which skin and other epidermal tissues (such as hair) are largely composed.

We focused on cetaceans—whales, dolphins, and porpoises—for several reasons. First, cetaceans lack the typical fur that covers the body of most mammals, including other marine mammals (e.g., seals and sea lions, sea otters, and polar bears). Although cetaceans retain a few hairs or vibrissae around the head and jaws, these are predominantly vestigial and probably serve as tactile sensory receptors [11]. This means that the skin of cetaceans is thin and susceptible to damage from sun, water-borne pollutants and irritants, and other chemical or physical effects [11]. Second, cetaceans have life spans that are very long relative to other sea creatures and even to other mammals; whales generally live from 50-100 years and possibly

even well over 100 years in the case of some species such as the bowhead whale [12]. Third, and most importantly, the largest, longest-lived whales are filter feeders that depend on an exclusively keratin-based oral filter to capture their food [13]. Thus not only the well-being of individual cetaceans but the entire ecology of mysticetes is based on proper integrity of keratin as a long-lasting, flexible yet strong and resilient protein [7]. How might OA affect the structural and mechanical properties of cetacean keratin on a microscopic and organismal/ecological level?

The oral filter of whales is made of baleen, a novel tissue (unique to mysticete whales) comprised solely of alpha keratin [14]. Baleen hangs from both sides of the palate in serially arranged triangular sheets [15]. Each rack consists of about 300 plates which are constructed like a sandwich of flat cortical sheets of keratin surrounding hollow, straw-like tubules as well as scattered intertubular keratin [16]. As with other keratinous integumentary specializations including hair, claws, hooves, and nails, baleen grows throughout life at its base [17] but the exposed portion consists entirely of dead cells which gradually erode and wear away, in the case of baleen via abrasive friction from food, flowing water, and perhaps contact with oral structures such as the tongue and lips [14]. This produces hair-like fringes as the interior horn tubules are exposed [16]. Together the plates and fringes form a dense, mat-like matrix that captures food particles ranging from tiny (1-20 mm) plankton to small (~10 cm) fish [13, 18]. Baleen grows about 10-20 cm per year (in all mysticete species) and a full sheet may represent 10 or more years of growth [12], such that if baleen were adversely affected by OA, there would be serious harmful consequences: namely, the baleen filter might break, soften, or otherwise be unable to function effectively in prey capture [19]. Likewise any acid-induced weakening of the keratin in any epidermal tissue of long-lived whales (from skin to cornea) would pose great risk to their health.

Alpha keratin is a fibrous or filamentous structural protein constructed as a dimeric polypeptide [7, 17]. There are often multiple disulfide bridges, hydrogen bonds, or other cross-linking bonds that stabilize the protein's tertiary structure, providing great strength while also maintaining flexibility [20-22]. Keratin's strength is essential in enabling integumentary structures to withstand strong forces and act as a barrier to keep pathogens outside the body. In integumentary structures such as baleen or claws, keratin's flexible, pliant strength [23] also plays a crucial role in these tissues' basic function (e.g., filtration) [17, 20-23].

For our laboratory-based investigation, we exposed samples of baleen plates and fringes to seawater with different pH regimes representing current and projected levels of ocean acidification, plus dramatically acidified seawater (to test extreme conditions) and standard, non-acidified seawater as a control. Following several months of exposure, we subjected the baleen samples to physical (mechanical) tests. We also performed chemical testing to determine to what extent, if any, the acid exposure broke down the keratin protein. We conducted similar tests on a small number of cetacean skin samples.

Materials And Methods

We obtained baleen samples from bowhead whales (*Balaena mysticetus*) and baleen and skin samples from North Atlantic right whales (*Eubalaena glacialis*). All tissues were collected in accordance with applicable statues. No animals were killed or harmed to collect baleen; no baleen was imported from outside USA. Bowhead baleen was obtained from Inupiat subsistence hunters in Utqiaġvik (Barrow), Alaska; right whale specimens came from animals that died naturally prior to or during stranding in three states (Virginia, Massachusetts, Florida) along the US Atlantic coast, with collection by the NOAA/NMFS Northeast Marine Mammal Stranding and Disentanglement Network or NOAA/NMFS Southeast Marine Mammal Stranding Network.

Bowhead and right whale baleen plates were cut into squares of 5 x 5 cm (4-7 mm thick). The baleen specimens were then placed in differentglass tanks each with 8 liters of artificial seawater (made with Instant Ocean), with the seawater's pH unchanged (i.e., natural, for control tests, with pH=8.12) or altered via introduction of acid. In preliminary trials we used 1 N formic acid, and in later trials we used 1N acetic acid. The pH levels in the four trial tanks were maintained at 5.9, 6.6., 6.9, and 7.6. Samples were kept fully submerged in the seawater for 12 weeks as pH was monitored (by checking with a pH meter) several times per week. A small fan pump was placed in each tank to circulate water and keep it from becoming stagnant (simulating a real swimming

whale). Because the water and acid evaporated (exacerbated by the water circulation), pH fluctuated slightly during the three months of the experimental trials (Table 1).

Table1. *pH of experimental and control trials*

Week	pH of Tank A	Tank B	Tank C	Tank D	Tank E
1	5.92	6.58	6.91	7.58	8.12
2	5.92	6.59	6.88	7.54	8.04
3	5.91	6.58	6.89	7.53	8.05
4	5.91	6.58	6.89	7.55	8.06
5	5.89	6.59	6.9	7.59	8.08
6	5.9	6.59	6.91	7.62	8.12
7	5.9	6.6	6.91	7.66	8.13
8	5.89	6.6	6.92	7.68	8.15
9	5.88	6.62	6.92	7.74	8.15
10	5.88	6.62	6.93	7.75	8.15
11	5.87	6.64	6.94	7.8	8.18
12	5.87	6.66	6.97	7.83	8.22
mean	5.89	6.60	6.91	7.66	8.12

At the end of the 12 week acid exposure period the samples were removed from the water tanks and dried, then subjected to material testing with a Mark-10 ES30 universal testing machine with M4-200 force gauge running Mesur^{TM} Gauge recording software (Copiague, NY USA).

A blunt or pointed tip was applied to each sample to test compression (up to 500 N of force), and samples were also clamped and pulled in compression (also to 500 N). At the same time, a Mitutoyo Digimatic micrometer (Kanagawa, Japan) was used to record displacement or deformation of the tissue due to the tensing or compressing force.

In one series of trials, the baleen specimens were compressed or tensed with a uniform stress (force) and the resulting strain (deformation) was measured. In another series, the forces (in N) required to fracture the specimens were recorded at the breaking point.

Because the tests were conducted at different times, not all experimental pH levels were used for each series of trials. Six samples were used per experimental treatment for each mechanical trial. We compared data from different trials with a simple t-test.

As a means to test potential acid-induced chemical breakdown of baleen, we also cut free, eroded hair-like fringes from the original baleen plates. These were cut into bundles of 30 g of tissue, with fringes cut to 4-10 mm, to increase the surface area of exposed keratin. For the chemical testing, we used hydrochloric acid, although with deuterium (heavy) chloride and deuterium-depleted water for nuclear magnetic resonance (NMR) testing. As with the acetic and formic acids, the deuterated hydrochloric acid was initially constituted to the same trial pH levels (~6, 7, 8) but we also sought to determine if keratin would deteriorate and break down under extreme, highly acidic conditions (pure deuterium chloric acid at pH=1 and diluted to pH=3). Samples were again isolated in acidic seawater, enclosed within sealed test tubesfor 12 weeks, with periodic NMR testing to check for presence of constituent amino acids at four week intervals (hence one-third and two-thirds of the way through the test as well as at the conclusion).

Right whale skin samples (3 x 3 cm, N=5 total) from a whale's dorsum (back, N=3) and from a cranial callosity (N=2) were also subjected to different acid levels for 6 weeks (pH 6.6, 7.6, and 8.1) and tested mechanically as well as examined microscopically to investigate potential acidification effects on skin.

Results

For the series of mechanical tests of tissue strength, one set of trials examined the effects of loading acid-exposed and control tissue samples in compression and tension, both at the same force (=220 N, or the equivalent of 50 pounds of force per square inch=50 psi), as shown in Tables 2 and 3.

There were no statistically significant differences (at the p=0.05 level) for any of the lowered pH conditions relative to the control (normal seawater pH) condition.

Table2. *Deformation (in mm) of baleen samples from 220 N compressive force.*

pH 5.9	pH 6.9	pH 8.12 (control)
2.85	2.39	2.58
1.86	1.67	2.00
3.02	2.43	2.23
3.46	2.3	2.05
2.79	2.45	3.05
1.98	2.49	2.52
Mean 2.66	Mean 2.25	Mean 2.38

Table3. *Deformation (in mm) of baleen samples from 220 N tensile force.*

pH 5.9	pH 6.9	pH 8.12 (control)
1.12	1.34	0.86
0.96	1.13	0.96
0.84	0.85	0.78
1.17	0.92	1.21
1.06	1.08	0.99
0.74	0.97	1.13
Mean 0.98	Mean 1.04	Mean 0.99

Another series of trials investigated the compressive and tensile forces required to reach the breaking point of baleen specimens (acid-exposed and control). The mean force required to break compressed baleen samples was 59.5 (±9.8 SEM) N for the pH 6.6 treatment, 60.12 (±9.7) N for the pH 7.6 treatment, and 69.36 (±6.8) N for the control pH 8.1 treatment, as shown in Figure 1. Although the acid-exposed samples broke under slightly less force—that is, they were slightly weakened—the effect was once again not statistically significant.

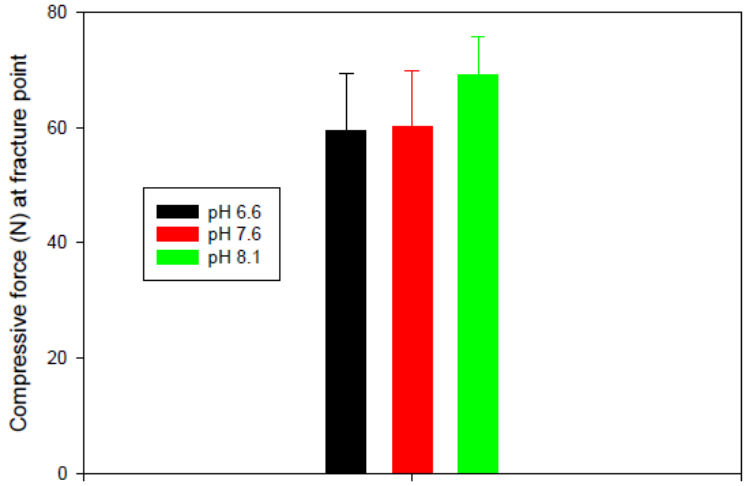

Figure1. *Force (in N) required to break baleen samples in compression (error bars=SEM).*

The baleen samples withstood much greater (2-3x) forces under tension (pulling). The mean force required to break tensed samples was 167 (±14.5 SEM) N for the pH 6.6 treatment, 181 (±17.8) N for the pH 7.6 treatment, and 192 (±16.1) N for the control pH 8.1 treatment, as shown in Figure 2. Again the acid-exposed samples broke under slightly less force, but again the effect was not statistically significant.

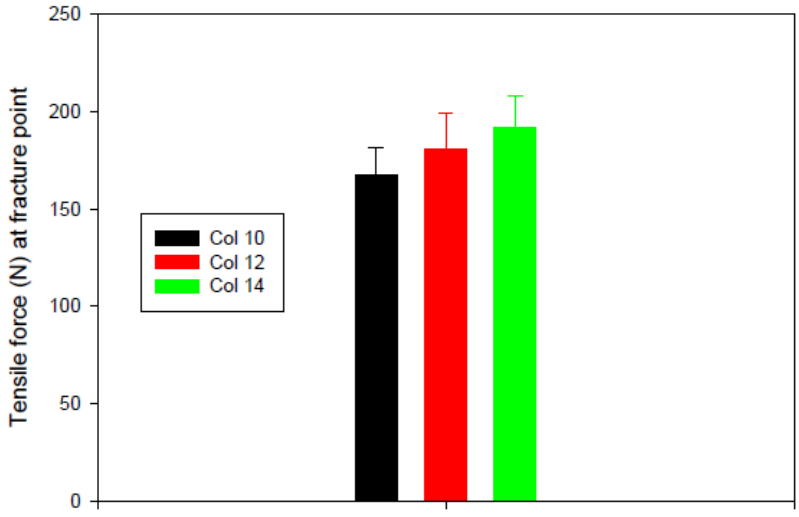

Figure2. *Force (in N) required to break baleen samples under tension (error bars=SEM).*

The nuclear magnetic resonance analysis investigated potential breakdown of keratin due to lower pH as would be caused by OA. If the protein in the baleen were broken down, testing would reveal different peaks in the NMR spectrum. These peaks would represent distinctly different amino acids released by the protein's deterioration [24], particularly lysine, arginine, or cysteine. Figure 3 shows NMR results from baleen placed in solutions of pH=1 (left of Fig. 3) and pH=3 (right). There are several more peaks in the pure acid (pH 1) sample, but none of these peaks are located with any of the amino acids we would expect to see from degenerative breakdown of keratin[24].

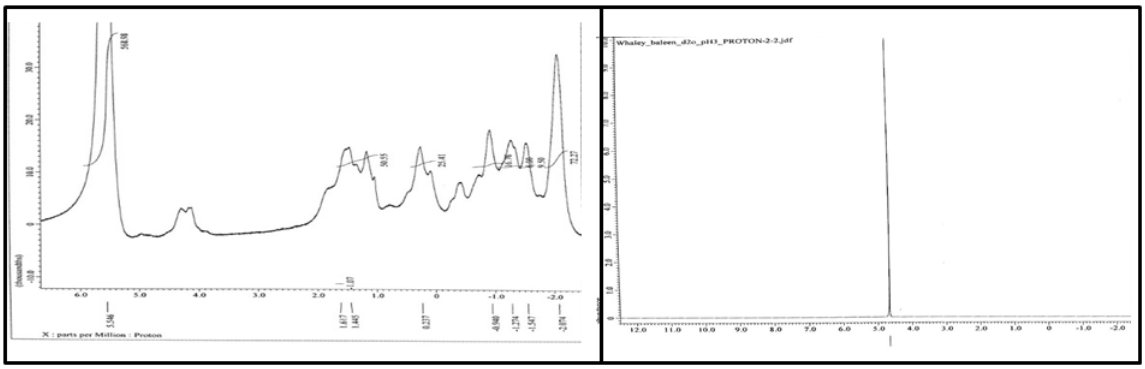

Figure3. *Nuclear magnetic resonance (NMR) spectra of baleen exposed to extremely low pH (pH=1 at left and pH=3 at right), neither of which show expected breakdown of keratin protein.*

The skin samples that were exposed to low pH (5.9 & 6.9) for six weeks also showed no remarkable features or notable differences in appearance or in strength from the control (pH=8.1) sample. All samples displayed keratinous fibers and keratinized cells in the stratified squamous epithelium of the epidermis, with thin (~ 10 µm) layers of hardened, cornified cells separated into layered, slightly separated sheets (Fig. 4). There was no notable discoloration or other alteration in appearance of cells placed in thin layers of seawater at normal or acidic pH (6.6 or 7.6) nor did the acid-treated skin samples have a demonstrably different texture or resistance to stretching.

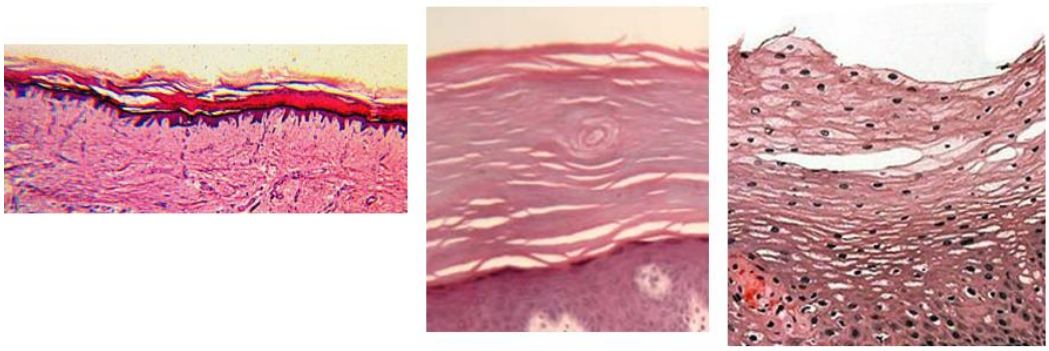

Figure4. *Microscopic view of right whale skin samples show no notable consequences of acid exposure (left, 40X view of skin from pH 5.9, center 100X from pH 6.9, right 100X from control pH 8.1).*

Discussion

We found slight but not statistically significant differences in baleen's mechanical (compressive and tensile) properties following the three month submergence in acidified seawater, even with greatly exaggerated H+ content (pH as low as 5.9) which is well beyond projected pH levels due to ocean acidification. We must note that the exceptionally low pH levels we investigated in this study do not reflect realistic projections for OA [3, 4]. However, the results demonstrate that acid-induced effects on keratin may not be likely even at exaggerated pH levels. This helps us to conclude that OA-related tissue changes, while still possible, are perhaps less likely than we expected at the outset of this study, which testifies to the remarkable strength and resilience of keratin [19, 25].

In addition, none of our NMR results (for any of the weeks, including the end of the study) indicated any acid-induced breakdown of keratin even at the lowest pH levels that we

tested. The peaks we obtained in the NMR spectra (Fig. 3) do not indicate chemical breakdown of keratin [24]; rather, they are due to the high levels of pure acid (pH=1 deuterated hydrochloric acid), perhaps with other, non-protein components (probably the test tube cap and Parafilm seal) released from the environment by the extreme acidity. More time (>12 weeks exposure) might have yielded a different result. Trials with pH>1 yielded no peaks, suggesting no breakdown of keratin due to acid exposure over the 12 week experimental period.

Our preliminary results therefore do not indicate substantial potential risk to oral or other integumentary keratin in cetaceans due to OA. However, it must be acknowledged that our trials lasted 12 weeks, which may not be long enough to show lasting effects of acidified seawater on keratin in baleen or skin. Due to the number of trials and conditions (e.g., our range of pH levels) we conducted, we have a sufficient sample size (with statistical power π=0.80, representing >80% confidence for significance at the 0.05 level)such that we feel confident in concluding there was no significant effect of acid exposure on the cetacean keratin samples we tested. Nonetheless our trials are continuing; we feel that OA remains a serious threat to cetaceans as well as to other marine species, and we are interested to investigate longer-term effects of acid exposure on keratin-based and other mammalian tissues. We also wish to test different acids and different biological samples or tissues. We had hoped the hair-like baleen fringes had sufficient surface area for chemical breakdown, and thus the fibers were cut into many short sections, but it is possible that even more area is needed to observe such effects.

Although we conclude that keratin is a strong, resilient protein, we remain concerned that OA poses a risk to whales and other marine mammals. Keratin can form an effective, impregnable barrier that sheds water in small amounts, but keratin is a hydrophilic material [26-28]. Higher levels of water exposure, as from total and prolonged submersion, allow water molecules to bind to and even penetrate the tubular and intertubular keratin of baleen [29]; if the water has a reduced pH from OA, the acid can therefore infiltrate the tissues more deeply and potentially cause greater internal damage, especially over long periods of time. This is of potentially greater consequence for the keratin in whale baleen, where plates persist for a decade or more [12], than in whale skin, which has a much shorter lifespan [11]. As noted previously, baleen is also essential for the filter feeding ecology of all mysticete whales. Studies of whale skin [11] indicate that these animals are also at risk of UV-induced sunburn, as well as to other threats posed by thinned or weakened skin, such as increased risk of skin laceration and abrasion or entry of bacteria, viruses, fungi, or other pathogens [11]. Other climate-related changes in the marine environment (such as increased ocean temperatures) might, when combined with OA-related pH changes, affect whales' cutaneous blood circulation and immune response, thermoregulation, and metabolism, especially given the large stores of crucial hypodermal blubber just below the skin [6].

Any chemical change in the seawater to which a whale's baleen is exposed could pose lasting damage to the whale's filter and thus to its dietary intake [13]. Although we were unable to document acid-related changes in the strength of baleen, it is possible that longer-term exposure could weaken baleen's internal structure, leading it to be less rigid or more likely to erode, both of which would substantially reduce baleen's effectiveness as an oral filter. We will continue to investigate the effects of seawater at reduced pH to explore other ways in which it might adversely affect the ecology of these endangered animals.

Conclusion

Ocean acidification remains a grave potential threat to all marine life, but our experiments did not demonstrate any acid-induced weakening leading to either mechanical or chemical breakdown of the fibrous keratin protein of which cetacean baleen and epidermal tissue are composed. Strength testing of baleen samples loaded in both compression and tension yielded similar deformation and fracture limits for control and lower-pH trials, and NMR analysis did not indicate constituent amino acids expected to be released by keratin following three month exposure to strongly or weakly acidic seawater.

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