# Emerging domoic acid concerns: Arctic food web contamination, age-associated susceptibility, and pathologies following chronic, low-level exposure

Alicia M. Hendrix

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2023

Reading Committee:

Thomas M. Burbacher, Co-Chair

David J. Marcinek, Co-Chair

Kathi A. Lefebvre

Program Authorized to Offer Degree:

Environmental & Occupational Health Sciences

University of Washington

## Abstract

Emerging domoic acid concerns: Arctic food web contamination, age-associated susceptibility, and pathologies following chronic, low-level exposure

Alicia M. Hendrix

Co-Chairs of the Supervisory Committee:
Thomas M. Burbacher
Environmental & Occupational Health Sciences
David J. Marcinek
Radiology

Human and wildlife communities depend on healthy marine environments to provide myriad resources and support. Harmful algal blooms (HABs), phenomena in which algae rapidly produce large amounts of biomass and often toxins, are becoming more frequent, widespread, and severe. This dissertation explores emerging concerns regarding domoic acid (DA), a HAB neurotoxin produced by diatoms in the genus *Pseudo-nitzschia* and known to cause toxicosis characterized by gastrointestinal (GI) distress, disorientation, amnesia, and mortality following consumption. The studies in chapters 2, 3, and 4 of this dissertation respectively address the potentially expanding geographic range of DA food web contamination, the interindividual susceptibilities that are of most concern for communities newly in contact with the toxin, and novel pathologies associated with new chronic exposure scenarios.

Chapter 2 of this thesis describes an analysis of GI samples from four species of Alaskan ice seal over an almost 10-year period. Samples were tested for DA and regression analyses were used to assess temporal trends. We found DA in GI samples from all of the four seal species tested (bearded, ringed, spotted, and ribbon seals), and in all three regions where samples were collected (Beaufort, Chukchi, and Bering Seas). We further detected an increasing trend of DA presence over the surveyed period in samples from bearded seals in the Bering Sea. Chapter 3 employs a mouse model to follow up on early reports that advanced age is associated with increased DA sensitivity. We administered one-time, symptomatic doses of DA to adult and aged female and male mice, observed seizure-related activity for 90 minutes following exposure, and then collected tissues for analysis of persistent DA levels. We report greater toxicity in aged mice, particularly aged females, and associated greater concentrations of DA in aged and female mouse tissue at 90 minutes post exposure. Finally, Chapter 4 shifts to focus on exposure scenarios in which consumers are regularly exposed to low levels of DA, an emergent threat as DA food web contamination becomes more ubiquitous. We exposed adult and aged female mice to 0.5 mg/kg bw DA thrice weekly for 14 weeks, assessed activity-related outcomes in Phenotyper cages, and then allowed a 10-week washout period before repeating tests. We also opportunistically measured cardiac function by echocardiography post exposure and again post washout. We found that DA exposure was associated with high mortality in aged mice, and with hyperactivity in adult mice, which did not appear to be significantly affected by washout. We found similar mild persistent effects of low dose DA on diastolic cardiac function. Collectively, these experiments contribute to the body of knowledge that informs effective protective measures for seafood consumers moving forward; we have identified geographic regions newly subject to DA threats, members of the population at greater risk for the health effects of

exposure, and both behavioral and cardiac pathways impacted by novel repeated, low-level exposure scenarios.

**Table of Contents**

**List of Tables**

**List of Figures**

**Acknowledgements**

I am humbled by the kindness, compassion, and generosity that I have been shown throughout my time in graduate school. Doctoral work is a unique experience, and I could not have survived it without extraordinary support along the way from mentors, teachers, staff, and peers in my program, and from my incredible family, both biological and chosen. I have recognized contributions from groups and individuals in each experimental chapter, but I would like to take time here at the outset to thank some of the people who have made this work and the graduate school experience that it sprang from possible.

Tom – I am truly lucky to have received your thoughtful feedback, your patient guidance, and your thorough edits throughout this undertaking. You amaze me with the level of attention that you deliver to each new question, and with your sincere interest in student well-being. I have learned so much from you, about how to conduct science, to share it in thoughtful ways, and to move through the world – academic and otherwise – with integrity. As you know, there were times in this experience where I very nearly didn't make it. You were extraordinary in those moments, and your care and humanity saved me. Thank you.

Dave – I am so grateful that I have had the chance to watch you work and run your lab. You model so many of the things that I aspire to: succinct writing, good – if rueful – cheer in the face of bureaucracy, constancy through new challenges. Your calm has grounded me through multiple milestones. I am so lucky to have been able to learn from you over these years, and I hope to carry some of your unflappability forward. Thank you.

Kathi – I started working with you on a weekend field excursion to the Olympic Peninsula seven years ago. I am so very thankful for that trip and everything that it set in motion. I'm in graduate school because of your passion, because you bring contagious energy to every

field, lab, and conference environment you enter, and because you appreciate neatly labeled tubes. I am so lucky to have a strong female scientist role model. Thank you. It's been my privilege to learn from you, both in the lab and in life.

To the UW, SPH, and DEOHS faculty, staff, and students I owe so much gratitude. My cohort came through for each other in study sessions, editing parties, and class discussions with stalwart solidarity. I know each of you will do – in fact are already doing – great things. The DEOHS Coders' group – especially Brian High and Chris Zuidema – were integral in both technical analytical support and buoying my spirits and confidence each time I struggled with a new R challenge. The members of the Marcinek, Lefebvre, and Burbacher labs have shaped my work through assistance on projects and feedback on my physical products, and I have been so lucky to share in their collaborative, thoughtful environments. Thank you also to Toby Cole of the Animal Behavior Core and to the animals and staff of the UW animal facilities.

Finally, not a word of this dissertation would have been possible without the extraordinary support of my family and friends. To my parents for their love and support, to my sister for her homecooked and delivered meals, to my grandfather for reminding me where we've come from, to my friends for parallel work parties, celebratory dinners, and emotional phone calls: thank you all from the bottom of my heart.

## Chapter 1: Introduction – Emergent concerns in domoic acid research

### 1.1. Oceans and Human Health

The year 2021 marked the beginning of the United Nations' (UN's) Decade of Ocean Science for Sustainable Development. This ten-year push aims to meet the UN's Sustainable Development Goals concerning human health and well-being as they intersect with global oceans [1]. Accompanying reports and agendas repeatedly acknowledge the critical role of marine and aquatic ecosystems in maintaining human livelihoods by providing both material and nonmaterial goods, e.g., food, trade, transport, and tourism, and esoteric benefits to cultural, mental, and physical health [1]. Anthropogenically-driven environmental changes that threaten the proper functioning of marine ecosystems could be disastrous for public health worldwide [1–3]. Oceans and Human Health (OHH) research unites fields as diverse as oceanography, marine ecology, medicine, environmental health science, public health, and economics to clarify and inform responses to such threats [2,4–6].

### 1.2. Harmful Algal Blooms and Their Toxins

Comprehensive OHH reports in Europe, the U.S., and Asia have pointed to harmful algal blooms (HABs) as a paradigmatic example of the interconnection of human and ocean health [1–3,5,7]. In these HAB events, microscopic algae rapidly produce large amounts of biomass and threaten fish, bird, and mammal health by clogging gills, depleting dissolved oxygen, and/or actively producing and releasing toxic compounds. Since the 1970s, when the First International Conference on Toxic Dinoflagellate Blooms was held in Boston, Massachusetts, HAB threats have increased in severity and frequency worldwide, with predictions that they will continue to grow in the future [8]. Today, they are recognized as a reflection of ecosystem instability and a

broad public health threat likely to worsen with climate change, eutrophication, and pollution [9,10].

### 1.3. The Harmful Algal Bloom Toxin Domoic Acid

Domoic acid (DA) is a small, polar molecule with a binding site similar to that of the neurotransmitter glutamate that is produced by many diatoms in the genus *Pseudo-nitzschia* and by closely related species [11–13]. Research into toxigenic environmental conditions predominantly focuses on stress-inducing nutrient limitation, fluctuating temperature, and salinity extremes, though a complete understanding of contributory environmental features remains elusive [14–24]. Once produced, DA is known to spread orally through marine food webs, contaminating filter feeding organisms and, in turn, higher level consumers [25–29]. Depuration time in marine species varies; some canonical vector species like razor clams (*Siliqua patula*) in the U.S. Pacific Northwest retain the toxin for long periods, allowing for bioaccumulation [26,30–33]. Because of this prolonged retention, food webs can contain prey with high levels of DA both during and after local *Pseudo-nitzschia* bloom events [30], and higher-level consumers can be exposed repeatedly throughout the year [34,35].

Following consumption of DA in sufficient quantities, the molecule can cross the blood-brain barrier and bind to glutamate receptors in the brain. Activation of the two non-N-methyl-D-aspartate receptor (non-NMDA-R) subtypes alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate (KA) receptors stimulates excitatory amino acid efflux [36–43]. This efflux in turn activates NMDA-Rs, exacerbating excitotoxicity and ultimately resulting in neuronal death through necrotic pathways and some degree of glial cell activation, most notably and consistently in the amygdala and hippocampus [44–49].

### 1.4. Observations of Domoic Acid Toxicosis in Marine Wildlife

Numerous DA poisonings have been suspected or confirmed in naturally exposed marine wildlife, including sea birds, California sea lions (CSLs; *Zalophus californianus*), southern sea otters (*Enhydra lutris nereis*), common dolphins (*Delphinus capensis*), humpback whales (*Megaptera novaeangliae*), blue whales (*Balaenoptera musculus*), Northern fur seals (*Callorhinus ursinus*), gray whales (*Eschrichtius robustus*), pygmy and dwarf sperm whales (*Kogia spp.*), Pacific walruses (*Odobenus rosmarus*), and minke whales (*Balaenoptera acutorostrata*) [50–54]. Wildlife researchers have pointed out that marine mammals susceptible to the toxin can serve as sentinel species for human health risks [34,55]. In fact, CSLs and southern sea otters are already serving this function. During May and June of 1998, sea lions presented with seizures, ataxia, and other neurological symptoms in and near Monterey Bay, California [56–58]. When it was shown that DA-containing *Pseudo-nitzschia* were present in the area at the time, and that DA was detectable both in the CSL prey northern anchovy (*Engraulis mordax*) and in CSL body fluids, the event was declared a confirmed CSL DA poisoning [58]. However, although monitoring designed to protect human health primarily focused on mussel DA levels at the time, Monterey's blue mussels (*Mytilus edulus*) were not found to have high DA levels during this incident; the event prompted changes in public health monitoring for DA contamination [58]. Since then, CSLs exposed naturally to DA have repeatedly informed both research and policy regarding human health threats of the toxin. Veterinary studies of CSLs have identified a distinct, long-lasting clinical syndrome characterized by persistent epilepsy following DA exposure [59], as well as a degenerative cardiomyopathy in CSLs exposed to both lethal and sublethal levels of DA [60]. Several reproductive and developmental exposure scenarios and outcomes of DA exposure likely relevant to humans have also been observed in

CSLs, including lactational and *in utero* exposure to DA, DA-associated miscarriages in pregnant CSLs, and an epileptic syndrome in offspring exposed to DA *in utero* [59,61–63]. Now, as climate change has worsened in the last decade, CSLs suffer DA poisonings annually off the Pacific Coast of California, heralding more serious DA threats for humans as well [64–66].

Southern sea otters found along the Californian coast have also shown increasing evidence of morbidity and mortality linked to DA. Since the first four reported cases of DA intoxication in southern sea otters in 2003, DA toxicosis has been linked to sea otter mortality in an increasing number of cases [67]. In part due to their high site fidelity, sea otters are at particularly high risk for health effects from local *Pseudo-nitzschia* blooms. In recent years, sea otter studies have reported an association between chronic DA exposure and cardiac disease, including overt cardiac lesions, myocarditis, and dilated cardiomyopathy [68–70]. This research may suggest new foci for concerns about chronic DA exposure in humans.

### 1.5. Domoic Acid Toxicity in Humans

Known as a medicinal compound in Japan since the 1950s, DA was not identified as a human neurotoxin until a widespread human poisoning event in 1987 [71,72]. Following the contamination of mussels from Prince Edward Island (PEI) in November of that year, hospitals began receiving patients with severe gastrointestinal distress, disorientation, and memory loss [71]. In total, over 150 people fell ill, and four ultimately died [71]. The term Amnesic Shellfish Poisoning (ASP) is now used to describe the clinical symptoms associated with acute exposure to high levels of DA [71]. Analysis of the event indicated that older consumers experienced greater rates of hospitalization and mortality following mussel consumption [71]. In patients under 65 years of age with severe symptoms, underlying conditions were common, and study authors

suggested compromised renal function was a driving factor in their susceptibility [71]. Since that time, however, follow-up research exploring possible age-associated susceptibility to DA toxicity has been limited.

Since the 1987 poisoning event, regulatory efforts have prevented incidents of acute DA toxicity in humans (see section 1.6). However, critical continuing work with a group of coastal American Indian communities in Washington State called the Communities Advancing the Studies of Tribal Nations across the Lifespan (CoASTAL) cohort highlights possible new human health concerns. Cohort members' DA exposure is approximated through estimations of their consumption of the well-known DA vector, razor clams [32], a cultural staple in many Salish Sea Indigenous diets [73]. CoASTAL results to date are subtle, but increasingly compelling. In 2016 an association was seen between chronic consumption of razor clams and some measures of memory, but these associations did not persist when DA measurements from specific beaches were used to fine-tune assumed DA exposure [74]. In 2018, a more focused study on a subset of the cohort reported an association between everyday working memory and high-level consumption of razor clams in both the last week and the last year [75]. Most recently, Stuchal et al. performed a DA dose-response analysis for persons with chronic or repeated low-level exposure and found a consistent association between declines in total learning recall memory and average DA dose per meal, regardless of whether a recovery or nonrecovery model was used [76]. All studies noted the need for further follow-up studies concerning possible human health impacts of chronic DA exposure.

### 1.6. Risk Assessments and Regulations Regarding Domoic Acid as a Public Health Threat

Following the catastrophic DA poisoning event in 1987, both the U.S. and Canada implemented public health programs in which DA levels in harvested shellfish would be monitored, and fisheries would close when detected DA levels exceeded 20 ppm [77]. This regulatory limit was derived from estimations of human exposure in the PEI incident, with a safety factor applied to protect vulnerable groups [77]. These regulatory approaches appear to have been successful in protecting the public from a widespread, acute DA poisoning event since their implementation. However, several research groups have conducted additional DA risk assessments since the 1980s, with sometimes conflicting conclusions about the adequacy of current regulatory limits for protecting human health. In 1996, Mariën administered consumption surveys to razor clam harvesters and used indicated exposure levels to conclude that a 20 ppm regulatory limit was appropriately protective against exceeding a tolerable daily intake (TDI) of 0.075 mg/kg bw (indicated by previous human and monkey studies) [78]. However, two years later, when a cohort of 24 cynomolgus monkeys demonstrated brain lesions following DA administration at levels close to 0.03 mg/kg bw, Slikker et al. argued that a DA regulatory limit of 6.4-12 ppm would be more appropriate [79]. Risk assessments since then have continued to, in some cases, support the 20 ppm level [80,81], and, in other cases, advocate for reducing the level to something more cautious [82]. Very recently, Stuchal et al. were the first to conduct a risk assessment specifically concerning chronic DA exposure [76]. Their data suggest that a "health based-limit for DA" at 2 ppm (an order of magnitude lower than current regulations) would be sufficient to protect regular seafood consumers from the total learning recall memory effects observed in some chronic DA consumers in the CoASTAL cohort [76]. The authors acknowledge that their study serves only as guidance for regulatory agencies, and that additional

factors should be considered in any final determinations [76]. In addition to the need for more risk assessments specifically concerned with chronic exposure, assessments focused on vulnerable groups including older adults and those with renal dysfunction should be a priority [83].

### 1.7. Dissertation Aims

Questions of DA's threat to public health are of increasing importance as oceans and coastal communities are transformed by climate change. This dissertation addresses three knowledge gaps identified in the summary above, namely 1) which geographic regions may be subject to novel DA contamination, 2) whether aging increases sensitivity to DA pathology, and 3) what pathologies are associated with chronic, low-level DA exposure.

The first aim of this dissertation (see Chapter 2) tests the hypothesis that there will be a significant increase in the levels of DA and a second HAB toxin, saxitoxin (STX), in Alaskan marine mammals over time. We quantified DA and STX levels in gastrointestinal samples from four species of ice seal harvested for subsistence purposes in the Bering, Chukchi, and Beaufort Seas from 2005-2019, and used regression analyses to test for increasing proportions of positive samples and/or increasing levels of toxin in positive samples over the period.

Chapter 2's assessment of new regions subject to DA contamination highlights the importance of characterizing DA-associated public health threats, and Chapter 3 begins to do this, namely by contributing to the body of knowledge regarding interindividual susceptibility to DA toxicosis. The laboratory work described in this chapter tests the hypotheses that aged animals exhibit increased sensitivity to acute DA excitotoxicity, and that sensitivity varies by sex. We injected female and male C57Bl/6 NIA adult (7-9 mo) and aged (25-28 mo) mice with

doses of DA between 0.5 and 2.5 mg/kg bw and observed activity and stereotypic epileptic behavior for 90 minutes, before sacrificing the animals and quantifying persistent DA in bodily tissues.

As outlined in the literature above and complemented by Chapter 2's work concerning food web contamination in Alaska, scenarios in which consumers are chronically or repeatedly exposed to low levels of DA are increasingly relevant. Additional work above, and Chapter 3's assessment of age-associated DA susceptibility, indicate that this may present a particular risk to older community members. Chapter 4 follows up on both of these concerns, testing the hypothesis that repeated, low-level DA exposure is associated with reversible behavioral and physiological pathological changes, which may be more severe in aged animals. We injected adult (13 mo) and aged (24 mo) female C57Bl/6 J mice with 0.5 mg/kg bw DA for 14 weeks before assessing behavior by Phenotyper cage monitoring. We then tested the reversibility of observed impairments by allowing a 10-week washout period for adult mice, and retesting behavioral endpoints. Secondary echocardiography analyses completed opportunistically at 13-week and 24-week timepoints were also able to evaluate cardiac function associated with DA exposures.

### 1.8. Research Impact

This project explores crucial knowledge gaps related to emergent DA threats. Chapter 2 brings awareness to Arctic and subarctic regions newly at risk for DA contamination. Chapter 3 is, to our knowledge, the first manuscript to evaluate DA toxicity in aged mice of both sexes following acute exposure. Finally, Chapter 4 explores pathologies in mice of different ages following chronic exposure to low levels of DA, a scenario more common in coastal

communities. These data may help identify community members for whom more ubiquitous DA exposure will be of greatest concern. Ultimately, the data presented in this dissertation will help to inform the development of effective and relevant protective public health measures as HAB toxin threats evolve.

### 1.9. References


1. Borja, A.; White, M.P.; Berdalet, E.; Bock, N.; Eatock, C.; Kristensen, P.; Leonard, A.; Lloret, J.; Pahl, S.; Parga, M.; et al. Moving Toward an Agenda on Ocean Health and Human Health in Europe. *Front Mar Sci* **2020**, *7*, 37, doi:10.3389/FMARS.2020.00037/BIBTEX.
2. Nash, K.L.; van Putten, I.; Alexander, K.A.; Bettiol, S.; Cvitanovic, C.; Farmery, A.K.; Flies, E.J.; Ison, S.; Kelly, R.; Mackay, M.; et al. Oceans and Society: Feedbacks between Ocean and Human Health. *Reviews in Fish Biology and Fisheries 2021* **2021**, 1–27, doi:10.1007/S11160-021-09669-5.
3. Short, R.E.; Cox, D.T.C.; Ling Tan, Y.; Bethel, A.; Eales, J.F.; Garside, R. Review of the Evidence for Oceans and Human Health Relationships in Europe: A Systematic Map. *Environ Int* **2021**, *146*, 106275, doi:10.1016/J.ENVINT.2020.106275.
4. Britton, E.; Domegan, C.; McHugh, P. Accelerating Sustainable Ocean Policy: The Dynamics of Multiple Stakeholder Priorities and Actions for Oceans and Human Health | Elsevier Enhanced Reader. *Mar Policy* **2021**, *124*, 104333.
5. Sandifer, P.A.; Keener, P.; Scott, G.I.; Porter, D.E. Oceans and Human Health and the New Blue Economy. In *Preparing a Workforce for the New Blue Economy*; Elsevier, 2021; pp. 213–236.
6. Tyson, F.L.; Rice, D.L.; Dearry, A. Connecting the Oceans and Human Health. *Environ Health Perspect* **2004**, *112*, A455–A456.
7. Kogure, K. Renewing the Health of the Oceans. *ICUS Commentary of Presentation Two* **2019**, 273–279.
8. Sellner, K.G.; Doucette, G.J.; Kirkpatrick, G.J. Harmful Algal Blooms: Causes, Impacts and Detection. *J Ind Microbiol Biotechnol* **2003**, *30*, 383–406, doi:10.1007/S10295-003-0074-9.
9. Epstein, P.R.; Chivian, E.; Frith, K. Nature's Services: Societal Dependence on Natural Ecosystems. *Plant Health Prog* **2003**, 10, doi:10.1094/PHP.
10. Young, N.; Sharpe, R.A.; Barciela, R.; Nichols, G.; Davidson, K.; Berdalet, E.; Fleming, L.E. Marine Harmful Algal Blooms and Human Health: A Systematic Scoping Review. *Harmful Algae* **2020**, *98*, 101901, doi:10.1016/j.hal.2020.101901.
11. Bates, S.S. Domoic-Acid-Producing Diatoms: Another Genus Added! *J Phycol* **2000**, *36*, 978–983, doi:10.1046/j.1529-8817.2000.03661.x.
12. Bates, S.S.; Trainer, V.L. The Ecology of Harmful Diatoms. In *Ecology of Harmful Algae*; Graneli, E., Turner, J.T., Eds.; Springer: Berlin, Heidelberg, 2006; pp. 81–93.

13. Kotaki, Y.; Koike, K.; Yoshida, M.; Chu, V.T.; Huyen, N.T.M.; Nguyen, C.H.; Fukuyo, Y.; Kodama, M. Domoic Acid Production in Nitzschia Sp. (Bacillariophyceae) Isolated from a Shrimp-Culture Pond in Do Son, Vietnam. *J Phycol* **2000**, *36*, 1057–1060, doi:10.1046/J.1529-8817.2000.99209.X.
14. Ayache, N.; Hervé, F.; Lundholm, N.; Amzil, Z.; Caruana, A.M.N. Acclimation of the Marine Diatom Pseudo-nitzschia Australis to Different Salinity Conditions: Effects on Growth, Photosynthetic Activity, and Domoic Acid Content. *J Phycol* **2020**, *56*, 97–109, doi:10.1111/jpy.12929.
15. Bretherton, L.; Hillhouse, J.; Bacosa, H.; Setta, S.; Genzer, J.; Kamalanathan, M.; Finkel, Z. v.; Quigg, A. Growth Dynamics and Domoic Acid Production of Pseudo-Nitzschia Sp. in Response to Oil and Dispersant Exposure. *Harmful Algae* **2019**, *86*, 55–63, doi:10.1016/j.hal.2019.05.008.
16. Fehling, J.; Davidson, K.; Bolch, C.J.; Bates, S.S. Growth and Domoic Acid Production by Pseudo-Nitzschia Seriata (Bacillariophyceae) under Phosphate and Silicate Limitation. *J Phycol* **2004**, *40*, 674–683, doi:10.1111/j.1529-8817.2004.03213.x.
17. Haroardóttir, S.; Wohlrab, S.; Hjort, D.M.; Krock, B.; Nielsen, T.G.; John, U.; Lundholm, N. Transcriptomic Responses to Grazing Reveal the Metabolic Pathway Leading to the Biosynthesis of Domoic Acid and Highlight Different Defense Strategies in Diatoms. *BMC Mol Biol* **2019**, *20*, 7, doi:10.1186/s12867-019-0124-0.
18. Lundholm, N.; Hansen, P.J.; Kotaki, Y. Effect of PH on Growth and Domoic Acid Production by Potentially Toxic Diatoms of the Genera Pseudo-Nitzschia and Nitzschia. *Mar Ecol Prog Ser* **2004**, *273*, 1–15.
19. Maldonado, M.T.; Hughes, M.P.; Rue, E.L.; Wells, M.L. The Effect of Fe and Cu on Growth and Domoic Acid Production by Pseudo-Nitzschia Multiseries and Pseudo-Nitzschia Australis. *Limnol Oceanogr* **2002**, *47*, 515–526, doi:10.4319/lo.2002.47.2.0515.
20. Martin-Jézéquel, V.; Calu, G.; Candela, L.; Amzil, Z.; Jauffrais, T.; Séchet, V.; Weigel, P. Effects of Organic and Inorganic Nitrogen on the Growth and Production of Domoic Acid by Pseudo-Nitzschia Multiseries and P. Australis (Bacillariophyceae) in Culture. *Mar Drugs* **2015**, *13*, 7067–7086, doi:10.3390/md13127055.
21. Pan, Y.; Subba Rao, D. v.; Mann, K.H. Changes in Domoic Acid Production and Cellular Chemical Composition of the Toxigenic Diatom Pseudo-Nitzschia Multiseries Under Phosphate Limitation. *J Phycol* **1996**, *32*, 371–381, doi:10.1111/j.0022-3646.1996.00371.x.
22. Pan, Y.; Bates, S.S.; Cembella, A.D. Environmental Stress and Domoic Acid Production by Pseudo-Nitzschia: A Physio-Logical Perspective. *Nat Toxins* **1998**, *6*, 127–135.
23. Sobrinho, B.F.; de Camargo, L.M.; Sandrini-Neto, L.; Kleemann, C.R.; Machado, E. da C.; Mafra, L.L. Growth, Toxin Production and Allelopathic Effects of Pseudo-Nitzschia Multiseries under Iron-Enriched Conditions. *Mar Drugs* **2017**, *15*, 331, doi:10.3390/md15100331.
24. Thorel, M.; Fauchot, J.; Morelle, J.; Raimbault, V.; le Roy, B.; Miossec, C.; Kientz-Bouchart, V.; Claquin, P. Interactive Effects of Irradiance and Temperature on Growth and Domoic Acid Production of the Toxic Diatom Pseudo-Nitzschia Australis (Bacillariophyceae). *Harmful Algae* **2014**, *39*, 232–241, doi:10.1016/j.hal.2014.07.010.
25. Bargu, S.; Powell, C.L.; Coale, S.L.; Busman, M.; Doucette, G.J.; Silver, M.W. Krill: A Potential Vector for Domoic Acid in Marine Food Webs. *Mar Ecol Prog Ser* **2002**, *237*, 209–216, doi:10.3354/MEPS237209.

26. Costa, P.R.; Rodrigues, S.M.; Botelho, M.J.; Sampayo, M.A. de M. A Potential Vector of Domoic Acid: The Swimming Crab Polybius Henslowii Leach (Decapoda-Brachyura). *Toxicon* **2003**, *42*, 135–141, doi:10.1016/S0041-0101(03)00107-7.
27. Costa, P.R.; Garrido, S. Domoic Acid Accumulation in the Sardine Sardina Pilchardus and Its Relationship to Pseudo-Nitzschia Diatom Ingestion. *Mar Ecol Prog Ser* **2004**, *284*, 261–268, doi:10.3354/MEPS284261.
28. Lefebvre, K.A.; Dovel, S.L.; Silver, M.W. Tissue Distribution and Neurotoxic Effects of Domoic Acid in a Prominent Vector Species, the Northern Anchovy Engraulis Mordax. *Mar Biol* **2001**, *138*, 693–700.
29. Powell, C.L.; Ferdin, M.E.; Busman, M.; Kvitek, R.G.; Doucette, G.J. Development of a Protocol for Determination of Domoic Acid in the Sand Crab (Emerita Analoga): A Possible New Indicator Species. *Toxicon* **2002**, *40*, 485–492, doi:10.1016/S0041-0101(01)00236-7.
30. Kvitek, R.G.; Goldberg, J.; Smith, G.; Doucette, G.; Silver, M. Domoic Acid Contamination within Eight Representative Species from the Benthic Food Web of Monterey Bay, California, USA. *Mar Ecol Prog Ser* **2008**, *367*, 35–47, doi:10.3354/meps07569.
31. Wekell, J.C.; Trainer, V.L.; Ayres, D.; Simons, D. A Study of Spatial Variability of Domoic Acid in Razor Clams: Recommendations for Resource Management on the Washington Coast. *Harmful Algae* **2002**, *1*, 35–43, doi:10.1016/S1568-9883(02)00004-5.
32. Wekell, J.C.; Gauglitz, E.J.; Barnett, H.J.; Hatfield, C.L.; Simons, D.; Ayres, D. Occurrence of Domoic Acid in Washington State Razor Clams (Siliqua Patula) during 1991-1993. *Nat Toxins* **1994**, *2*, 197–205, doi:10.1002/NT.2620020408.
33. Wohlgeschaffen, G.D.; Mann, K.H.; Subba Rao, D. v.; Pocklington, R. Dynamics of the Phycotoxin Domoic Acid: Accumulation and Excretion in Two Commercially Important Bivalves. *Journal of Applied Phycology 1992 4:4* **1992**, *4*, 297–310, doi:10.1007/BF02185786.
34. Hendrix, A.M.; Lefebvre, K.A.; Quakenbush, L.; Bryan, A.; Stimmelmayr, R.; Sheffield, G.; Wisswaesser, G.; Willis, M.L.; Bowers, E.K.; Kendrick, P.; et al. Ice Seals as Sentinels for Algal Toxin Presence in the Pacific Arctic and Subarctic Marine Ecosystems. *Mar Mamm Sci* **2021**, *37*, 1292–1308, doi:10.1111/MMS.12822.
35. Lefebvre, K.A.; Quakenbush, L.; Frame, E.; Huntington, K.B.; Sheffield, G.; Stimmelmayr, R.; Bryan, A.; Kendrick, P.; Ziel, H.; Goldstein, T.; et al. Prevalence of Algal Toxins in Alaskan Marine Mammals Foraging in a Changing Arctic and Subarctic Environment. *Harmful Algae* **2016**, *55*, 13–24, doi:10.1016/j.hal.2016.01.007.
36. Berman, F.W.; LePage, K.T.; Murray, T.F. Domoic Acid Neurotoxicity in Cultured Cerebellar Granule Neurons Is Controlled Preferentially by the NMDA Receptor Ca2+ Influx Pathway. *Brain Res* **2002**, *924*, 20–29, doi:10.1016/S0006-8993(01)03221-8.
37. Berman, F.W.; Murray, T.F. Domoic Acid Neurotoxicity in Cultured Cerebellar Granule Neurons Is Mediated Predominantly by NMDA Receptors That Are Activated as a Consequence of Excitatory Amino Acid Release. *J Neurochem* **2002**, *69*, 693–703, doi:10.1046/j.1471-4159.1997.69020693.x.
38. Hampson, D.R.; Manalo, J.L. The Activation of Glutamate Receptors by Kainic Acid and Domoic Acid. *Nat Toxins* **1998**, *6*, 153–158.
39. Larm, J.A.; Beart, P.M.; Cheung, N.S. Neurotoxin Domoic Acid Produces Cytotoxicity via Kainate- and Ampa-Sensitive Receptors in Cultured Cortical Neurones. *Neurochem Int* **1997**, *31*, 677–682, doi:10.1016/S0197-0186(97)00030-2.

40. Novelli, A.; Kispert, J.; Fernández-Sánchez, M.T.; Torreblanca, A.; Zitko, V. Domoic Acid-Containing Toxic Mussels Produce Neurotoxicity in Neuronal Cultures through a Synergism between Excitatory Amino Acids. *Brain Res* **1992**, *577*, 41–48, doi:10.1016/0006-8993(92)90535-H.
41. Pérez-Gómez, A.; Tasker, R.A. Domoic Acid As a Neurotoxin. *Handbook of Neurotoxicity* **2021**, 1–25, doi:10.1007/978-3-030-71519-9_87-1.
42. Tasker, R.A.R.; Perry, M.A.; Doucette, T.A.; Ryan, C.L. NMDA Receptor Involvement in the Effects of Low Dose Domoic Acid in Neonatal Rats. *Amino Acids 2005 28:2* **2005**, *28*, 193–196, doi:10.1007/S00726-005-0167-Z.
43. Tasker, R.A.R.; Strain, S.M. Synergism between NMDA and Domoic Acid in a Murine Model of Behavioural Neurotoxicity. *Neurotoxicology* **1998**, *19*, 593–597.
44. Ananth, C.; Thameem Dheen, S.; Gopalakrishnakone, P.; Kaur, C. Domoic Acid-Induced Neuronal Damage in the Rat Hippocampus: Changes in Apoptosis Related Genes (Bcl-2, Bax, Caspase-3) and Microglial Response. *J Neurosci Res* **2001**, *66*, 177–190, doi:10.1002/JNR.1210.
45. Gill, S.S.; Hou, Y.; Ghane, T.; Pulido, O.M. Regional Susceptibility to Domoic Acid in Primary Astrocyte Cells Cultured from the Brain Stem and Hippocampus. *Marine Drugs 2008, Vol. 6, Pages 25-38* **2008**, *6*, 25–38, doi:10.3390/MD6010025.
46. Pulido, O.M. Domoic Acid Toxicologic Pathology: A Review. *Mar Drugs* **2008**, *6*, 180–219, doi:10.3390/md20080010.
47. Sutherland, R.J.; Hoesing, J.M.; Whishaw, I.Q. Domoic Acid, an Environmental Toxin, Produces Hippocampal Damage and Severe Memory Impairment. *Neurosci Lett* **1990**, *120*, 221–223, doi:10.1016/0304-3940(90)90043-9.
48. Tasker, R.A.R.; Connell, B.J.; Strain, S.M. Pharmacology of Systemically Administered Domoic Acid in Mice. *Can J Physiol Pharmacol* **1991**, *69*, 378–382, doi:10.1139/y91-057.
49. Tryphonas, L.; Truelove, J.; Nera, E.; Iverson, F. Acute Neurotoxicity of Domoic Acid in the Rat. *Toxicol Pathol* **1990**, *18*, 1–9, doi:10.1177/019262339001800101.
50. Beltran, A.S.; Palafox-Uribe, M.; Grajales-Montel, J.; Cruz-Villacorta, A.; Ochoa, J.L. Sea Bird Mortality at Cabo San Lucas, Mexico: Evidence That Toxic Diatom Blooms Are Spreading. *Toxicon* **1997**, *35*, 447–453.
51. Fire, S.E.; Wang, Z.; Berman, M.; Langlois, G.W.; Morton, S.L.; Sekula-Wood, E.; Benitez-Nelson, C.R. Aquatic Mammals. **2010**, *36*, 342–350, doi:10.1578/AM.36.4.2010.342.
52. Fire, S.E.; Wang, Z.; Leighfield, T.A.; Morton, S.L.; McFee, W.E.; McLellan, W.A.; Litaker, R.W.; Tester, P.A.; Hohn, A.A.; Lovewell, G.; et al. Domoic Acid Exposure in Pygmy and Dwarf Sperm Whales (Kogia Spp.) from Southeastern and Mid-Atlantic U.S. Waters. *Harmful Algae* **2009**, *8*, 658–664.
53. Lefebvre, K.A.; Bargu, S.; Kieckhefer, T.; Silver, M.W. From Sanddabs to Blue Whales: The Pervasiveness of Domoic Acid. *Toxicon* **2002**, *40*, 971–977, doi:10.1016/S0041-0101(02)00093-4.
54. van Dolah, F.M.; Doucette, G.J.; Gulland, F.M.D.; Rowles, T.L.; Bossart, G.D. Impacts of Algal Toxins on Marine Mammals. In *Toxicology of Marine Mammals*; Vos, J.G., Bossart, G.D., Fournier, M., O'Shea, T.J., Eds.; Taylor & Francis: London and New York, 2003; Vol. 3, pp. 247–269 ISBN 0-415-23914-1.
55. Bossart, G.D. Marine Mammals as Sentinel Species for Oceans and Human Health. *Vet Pathol* **2011**, *48*, 676–690, doi:10.1177/0300985810388525.

56. Gulland, F. *Domoic Acid Toxicity in California Sea Lions (Zalophus Californianus) Stranded Along the Central California Coast, May-October 1998*; NOAA Technical Memorandum NMFS-OPR-17, 2000;
57. Lefebvre, K.A.; Powell, C.L.; Busman, M.; Doucette, G.J.; Moeller, P.D.R.; Silver, J.B.; Miller, P.E.; Hughes, M.P.; Singaram, S.; Silver, M.W.; et al. Detection of Domoic Acid in Northern Anchovies and California Sea Lions Associated with an Unusual Mortality Event. *Nat Toxins* **1999**, *7*, 85–92, doi:10.1002/(SICI)1522-7189(199905/06)7:3<85::AID-NT39>3.0.CO;2-Q.
58. Scholin, C.A.; Gulland, F.; Doucette, G.J.; Benson, S.; Busman, M.; Chavez, F.P.; Cordaro, J.; DeLong, R.; de Vogelaere, A.; Harvey, J.; et al. Mortality of Sea Lions along the Central California Coast Linked to a Toxic Diatom Bloom. *Nature* **2000**, *403*, 80–84, doi:10.1038/47481.
59. Goldstein, T.; Mazet, J.A.K.; Zabka, T.S.; Langlois, G.; Colegrove, K.M.; Silver, M.; Bargu, S.; van Dolah, F.; Leighfield, T.; Conrad, P.A.; et al. Novel Symptomatology and Changing Epidemiology of Domoic Acid Toxicosis in California Sea Lions (Zalophus Californianus): An Increasing Risk to Marine Mammal Health. *Proceedings of the Royal Society B: Biological Sciences* **2008**, *275*, 267–276, doi:10.1098/rspb.2007.1221.
60. Zabka, T.S.; Goldstein, T.; Cross, C.; Mueller, R.W.; Kreuder-Johnson, C.; Gill, S.; Gulland, F.M.D. Characterization of a Degenerative Cardiomyopathy Associated with Domoic Acid Toxicity in California Sea Lions (Zalophus Californianus). *Vet Pathol* **2009**, *46*, 105–119, doi:10.1354/vp.46-1-105.
61. Lefebvre, K.A.; Hendrix, A.; Halaska, B.; Duignan, P.; Shum, S.; Isoherranen, N.; Marcinek, D.J.; Gulland, F. Domoic Acid in California Sea Lion Fetal Fluids Indicates Continuous Exposure to a Neuroteratogen Poses Risks to Mammals. *Harmful Algae* **2018**, *79*, doi:10.1016/j.hal.2018.06.003.
62. Ramsdell, J.S.; Zabka, T.S. In Utero Domoic Acid Toxicity: A Fetal Basis to Adult Disease in the California Sea Lion (Zalophus Californianus). *Mar Drugs* **2008**, *6*, 262–290, doi:10.3390/md20080013.
63. Rust, L.; Gulland, F.; Frame, E.; Lefebvre, K.A. Domoic Acid in Milk of Free Living California Marine Mammals Indicates Lactational Exposure Occurs. *Mar Mamm Sci* **2014**, *30*, 1272–1278, doi:10.1111/mms.12117.
64. Bargu, S.; Silver, M.; Goldstein, T.; Roberts, K.; Gulland, F. Complexity of Domoic Acid-Related Sea Lion Strandings in Monterey Bay, California: Foraging Patterns, Climate Events, and Toxic Blooms. *Mar Ecol Prog Ser* **2010**, *418*, 213–222, doi:10.3354/meps08816.
65. Bargu, S.; Goldstein, T.; Roberts, K.; Li, C.; Gulland, F. Pseudo-Nitzschia Blooms, Domoic Acid, and Related California Sea Lion Strandings in Monterey Bay, California. *Mar Mamm Sci* **2012**, *28*, 237–253, doi:10.1111/j.1748-7692.2011.00480.x.
66. Greig, D.J.; Gulland, F.; Kreuder, C. A Decade of Live California Sea Lion (Zalophus Californianus) Strandings Along the Central California Coast: Causes and Trends, 1991-2000. **2005**, doi:10.1578/AM.31.1.2005.11.
67. Kreuder, C.; Miller, M.A.; Jessup, D.A.; Lowenstine, L.J.; Harris, M.D.; Ames, J.A.; Carpenter, T.E.; Conrad, P.A.; Mazet, J.A.K. Patterns of Mortality in Southern Sea Otters (Enhydra Lutris Nereis) from 1998-2001. *J Wildl Dis* **2003**, *39*, 495–509, doi:10.7589/0090-3558-39.3.495.

68. Kreuder, C.; Miller, M.A.; Lowenstine, L.J.; Conrad, P.A.; Carpenter, T.E.; Jessup, D.A.; Mazet, J.A.K. Evaluation of Cardiac Lesions and Risk Factors Associated with Myocarditis and Dilated Cardiomyopathy in Southern Sea Otters (Enhydra Lutris Nereis). *Am J Vet Res* **2005**, *66*, 289–299, doi:10.2460/ajvr.2005.66.289.
69. Moriarty, M.E.; Miller, M.A.; Murray, M.J.; Duignan, P.J.; Gunther-Harrington, C.T.; Field, C.L.; Adams, L.M.; Schmitt, T.L.; Johnson, C.K. Exploration of Serum Cardiac Troponin I as a Biomarker of Cardiomyopathy in Southern Sea Otters (Enhydra Lutris Nereis). *Am J Vet Res* **2021**, *82*, 529–537, doi:10.2460/AJVR.82.7.529.
70. Miller, M.A.; Moriarty, M.E.; Duignan, P.J.; Zabka, T.S.; Dodd, E.; Batac, F.I.; Young, C.; Reed, A.; Harris, M.D.; Greenwald, K.; et al. Clinical Signs and Pathology Associated With Domoic Acid Toxicosis in Southern Sea Otters (Enhydra Lutris Nereis). *Front Mar Sci* **2021**, *0*, 208, doi:10.3389/FMARS.2021.585501.
71. Perl, T.M.; Bédard, L.; Kosatsky, T.; Hockin, J.C.; Todd, E.C.D.; Remis, R.S. An Outbreak of Toxic Encephalopathy Caused by Eating Mussels Contaminated with Domoic Acid. *New England Journal of Medicine* **1990**, *322*, 1775–1780, doi:10.1056/NEJM199006213222504.
72. Takemoto, T.; Daigo, K. Constituents of Chondria Armata. *Chem Pharm Bull (Tokyo)* **1958**, *6*, 578b–5580, doi:10.1248/CPB.6.578B.
73. Crosman, K.M.; Petrou, E.L.; Rudd, M.B.; Tillotson, M.D.; Crosman, K.M.; Petrou, E.L.; Rudd, M.B.; Tillotson, M.D. Clam Hunger and the Changing Ocean: Characterizing Social and Ecological Risks to the Quinault Razor Clam Fishery Using Participatory Modeling. *Ecology and Society, Published online: May 16, 2019 | doi:10.5751/ES-10928-240216* **2019**, *24*, doi:10.5751/ES-10928-240216.
74. Grattan, L.M.; Boushey, C.; Tracy, K.; Trainer, V.L.; Roberts, S.M.; Schluterman, N.; Morris, J.G. The Association between Razor Clam Consumption and Memory in the CoASTAL Cohort. *Harmful Algae* **2016**, *57*, 20–25, doi:10.1016/j.hal.2016.03.011.
75. Grattan, L.M.; Boushey, C.J.; Liang, Y.; Lefebvre, K.A.; Castellon, L.J.; Roberts, K.A.; Toben, A.C.; Morris, J.G. Repeated Dietary Exposure to Low Levels of Domoic Acid and Problems with Everyday Memory: Research to Public Health Outreach. *Toxins (Basel)* **2018**, *10*, doi:10.3390/toxins10030103.
76. Stuchal, L.D.; Grattan, L.M.; Portier, K.M.; Kilmon, K.A.; Manahan, L.M.; Roberts, S.M.; Morris, J.G. Dose-Response Assessment for Impaired Memory from Chronic Exposure to Domoic Acid among Native American Consumers of Razor Clams. *Regulatory Toxicology and Pharmacology* **2020**, *117*, 104759, doi:10.1016/j.yrtph.2020.104759.
77. Wekell, J.C.; Hurst, J.; Lefebvre, K.A. The Origin of the Regulatory Limits for PSP and ASP Toxins in Shellfish. *J Shellfish Res* **2004**, *23*, 927–930.
78. Mariën, K. Establishing Tolerable Dungeness Crab (Cancer Magister) and Razor Clam (Siliqua Patula) Domoic Acid Contaminant Levels. *Environ Health Perspect* **1996**, *104*, 1230, doi:10.1289/EHP.104-1469507.
79. Slikker, W.; Scallet, A.C.; Gaylor, D.W. Biologically-Based Dose–Response Model for Neurotoxicity Risk Assessment. *Toxicol Lett* **1998**, *102–103*, 429–433, doi:10.1016/S0378-4274(98)00335-X.
80. Kumar, K.P.; Kumar, S.P.; Nair, G.A. Risk Assessment of the Amnesiac Shellfish Poison, Domoic Acid, on Animals and Humans. *J Environ Biol* **2009**, *30*, 319–325, doi:10.1016/j.automatica.2013.03.005.

81. Toyofuku, H. Joint FAO/WHO/IOC Activities to Provide Scientific Advice on Marine Biotoxins (Research Report). *Mar Pollut Bull* **2006**, *52*, 1735–1745.
82. Alexander, J.; Benford, D.; Boobis, A.; Ceccatelli, S.; Cravedi, J.-P.; di Domenico, A.; Doerge, D.; Dogliotti, E.; Edler, L.; Farmer, P.; et al. Marine Biotoxins in Shellfish – Domoic Acid. *EFSA Journal* **2009**, *7*, 1181, doi:10.2903/J.EFSA.2009.1181.
83. Petroff, R.; Hendrix, A.; Shum, S.; Grant, K.S.; Lefebvre, K.A.; Burbacher, T.M. Public Health Risks Associated with Chronic, Low-Level Domoic Acid Exposure: A Review of the Evidence. *Pharmacol Ther* **2021**, *227*, doi:10.1016/J.PHARMTHERA.2021.107865.

**Chapter 2: Ice seals as sentinels for algal toxin presence in the Pacific Arctic and subarctic marine ecosystems**

*This chapter has been published in Marine Mammal Science. The authors are:*

Alicia M. Hendrix[1], Kathi A. Lefebvre[2*] Lori Quakenbush[3], Anna Bryan[3], Raphaela Stimmelmayr[4,5], Gay Sheffield[6], Gabriel Wisswaesser[2], Maryjean L. Willis[2], Emily K. Bowers[2], Preston Kendrick[7], Elizabeth Frame[8], Thomas M. Burbacher[1], David J. Marcinek[7]

[1]Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA

[2]Environmental and Fisheries Science Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, Seattle, WA

[3]Arctic Marine Mammal Program, Alaska Department of Fish and Game, Fairbanks, AK

[4]North Slope Borough Department of Wildlife Management, Utqiaġvik AK

[5]Institute of Arctic Biology, University of Alaska Fairbanks, Fairbanks, AK

[6]Alaska Sea Grant Marine Advisory Program, University of Alaska Fairbanks, Nome, AK

[7]Departments of Radiology and Pathology and Bioengineering, University of Washington Medical School, Seattle, WA

[8]Aquatic Toxicology Unit, King County Environmental Laboratory, Seattle, WA

## 2.1. Abstract

Domoic acid (DA) and saxitoxin (STX)-producing algae are present in Alaskan seas, presenting exposure risks to marine mammals that may be increasing due to climate change. To investigate potential increases in exposure risks to four pagophilic ice seal species (*Erignathus barbatus*, bearded seals; *Pusa hispida*, ringed seals; *Phoca largha*, spotted seals; and *Histriophoca fasciata*, ribbon seals), this study analyzed samples from 998 seals harvested for subsistence purposes in western and northern Alaska during 2005-2019 for DA and STX. Both toxins were detected in bearded, ringed, and spotted seals, though no clinical signs of acute neurotoxicity were reported in harvested seals. Bearded seals had the highest prevalence of each toxin, followed by ringed seals. Bearded seal stomach content samples from the Bering Sea showed a significant increase in DA prevalence with time (logistic regression; $p=0.004$). These findings are consistent with predicted northward expansion of DA-producing algae. A comparison of paired samples taken from the stomachs and colons of 15 seals found that colon content consistently had higher concentrations of both toxins. Collectively, these results suggest that ice seals, particularly bearded seals (benthic foraging specialists), are suitable sentinels for monitoring HAB prevalence in the Pacific Arctic and subarctic.

**Keywords:** domoic acid, exposure risks, harmful algal blooms, marine mammals, saxitoxin

## 2.2. Introduction

### *2.2.1. Changing Ocean Conditions*

Arctic and subarctic seas are experiencing dramatic changes in the persistence, extent, and quality of sea ice due to changing weather patterns and warming ocean temperatures. This is particularly true in the Alaskan Arctic (N. R. Bates et al., 2014; Stevenson & Lauth, 2019) where inputs of Pacific water advected through the Bering Strait are fresher, warmer, and higher in volume (Hu et al., 2012) and where upwelling-favorable winds have also increased (Pickart et al., 2013). Warmer air temperatures and consequently larger negative air-sea heat fluxes have compounded conditions, leading to earlier snowmelt and elevated radiative forcing (Bintanja & van der Linden, 2013; Johannessen et al., 2004; Stone et al., 2002; Turner & Overland, 2009). These changes have affected the ecology and biogeography of species at multiple trophic levels (Capotondi et al., 2012; Stevenson & Lauth, 2019; Tremblay & Gagnon, 2009), and as a result, many temperate organisms are predicted to increase their distribution into or increase their numbers within Arctic waters. In the context of impacts to human, wildlife, and ecosystem health, Anderson et al. (2018) argue that one of the most significant emerging threats is the expansion of harmful algal bloom (HAB) species, particularly diatoms of the genus *Pseudo-nitzschia* and the dinoflagellate *Alexandrium catenella* that produce the potent neurotoxins domoic acid (DA) and saxitoxin (STX), respectively.

### *2.2.2. Health Effects of Harmful Algal Blooms*

Harmful algal blooms of DA-producing *Pseudo-nitzschia* and STX-producing *Alexandrium* species are common throughout the temperate world oceans and cause adverse human and wildlife health impacts and mortality. In humans, acute exposure leads to neurologic

illnesses known as Amnesic Shellfish Poisoning, caused by DA (S. S. Bates, 2000; S. S. Bates & Trainer, 2006; Berman & Murray, 2002; Perl et al., 1990; Todd, 1993), and Paralytic Shellfish Poisoning, caused by the suite of paralytic shellfish toxins (PSTs) including STX (Etheridge, 2010; Usup et al., 1994). Both toxins accumulate in filter-feeding marine organisms and are transferred through food webs with significant health consequences to animals at multiple trophic levels (Cembella & Desbiens, 1994; Kvitek et al., 2008; Lefebvre, Bargu, et al., 2002; Lefebvre et al., 2010; Lefebvre, Silver, et al., 2002; Scholin et al., 2000; White, 1980, 1981). Domoic acid exposure causes illness, stranding, and death in seabirds and marine mammals (Fritz et al., 1992; Gulland et al., 2005; Peery et al., 2006; Work et al., 1993). Persistent effects of recurrent DA exposures also lead to long-term neurotoxic effects and epilepsy in California sea lions (*Zalophus californianus*) (Cook et al., 2015; Goldstein et al., 2008). Exposures to STX also cause illness and death in marine mammals, although less frequently than those reported for DA. However, STX has been documented to cause massive kills of fish and invertebrates (Shumway, 1990; White, 1980, 1981), and has been linked to a mass mortality of humpback whales (*Megaptera novaeangliae*) off the eastern U.S. coast of Cape Cod, Massachusetts (Geraci et al., 1989). Together, these algal toxins result in significant economic losses in coastal communities relying on commercial and recreational seafood harvesting (C. R. Anderson et al., 2010; D. M. Anderson et al., 2000; Shumway, 1990; Trainer et al., 2007).

### *2.2.3. Marine Mammal Exposure to Harmful Algal Bloom Toxins*

In the last two decades, almost half of the marine mammal unusual mortality events in the contiguous U.S. have been attributable to algal toxin exposure (Flewelling et al., 2005; Gulland & Hall, 2007; Landsberg et al., 2014; Scholin et al., 2000), and there is concern that wildlife

exposure to HAB toxins may be growing. Domoic acid is known to be particularly common on the west coast of the contiguous U.S., where the first documented marine mammal DA poisoning event occurred in Monterey Bay, California in 1998. During this event, several hundred California sea lions exhibited seizures and/or died over a short period due to consumption of DA-contaminated anchovies (Gulland, 2000; Lefebvre et al., 1999; Scholin et al., 2000). Since then, dozens to hundreds of sea lions have been affected annually in coastal California (Bargu et al., 2010). In 2015, DA-induced seizures were first observed in sea lions north of California; in Long Beach, Washington during the largest recorded *Pseudo-nitzschia* bloom in coastal waters of North America (McCabe et al., 2016). This bloom was linked to a warm water anomaly that affected oceanic waters northward into the Gulf of Alaska, providing evidence for a potential northward expansion of conditions favorable for *Pseudo-nitzschia* growth (Zhu et al., 2017). Saxitoxin has been a marine mammal health concern since suspected poisonings in the late 1980s affected humpback whales in New England and sea otters (*Enhydra lutris*) in Alaska (DeGange & Vacca, 1989; Geraci et al., 1989; Landsberg et al., 2014). In a recent analysis of HAB events on the Pacific coast of Canada from 1988 to 2017, it was found that STX events occurred on the Canadian Pacific coast with regularity, while DA events occurred infrequently (McKenzie et al., 2020). Algal toxins have been reported in Alaskan Arctic marine mammals, however, algal toxin exposure has not been definitively linked to morbidity and mortality events in the region, and few data exist regarding these events in Alaskan pagophilic seal species (Lefebvre et al., 2016).

### *2.2.4. Ice Seal Exposure to Harmful Algal Bloom Toxins*

Bearded (*Erignathus barbatus*), ringed (*Pusa hispida*), spotted (*Phoca largha*), and ribbon (*Histriophoca fasciata*) seals represent critical components of the Pacific Arctic and

subarctic marine ecosystems. Collectively referred to as ice seals due to the integral role that ice plays as a substrate for pupping, nursing, and molting, these seals are an important subsistence resource for coastal Alaska Native communities in western and northern Alaska (Nelson et al., 2019). They are also an important component of the Arctic marine ecosystem. In December of 2012, NOAA Fisheries listed ringed and bearded seals as threatened under the Endangered Species Act, citing climate change and resultant sea ice declines as significant threats to the seals' survival (U.S. Federal Register, 2012a, 2012b). Previous analyses of gastrointestinal (GI) samples collected during 2006-2013 detected DA in all four of these ice seal species, and STX in all species except ribbon seals (Lefebvre et al., 2016). As environmental conditions in western and northern Alaska continue to transition, the potential for HAB toxins to increase in prevalence and concentration in the Bering and Chukchi Seas is an increasing health threat for ice seals (D. M. Anderson et al., 2018; Laidre et al., 2015). The objective of this study was to quantify DA and STX prevalence and assess temporal trends therein in four ice seal species in the Bering, Chukchi, and Beaufort Seas. Gastrointestinal samples were collected during 2005-2019 in partnership with coastal Alaska Native communities that harvest ice seals for subsistence purposes (Nelson et al., 2019).

## 2.3. Methods

### *2.3.1. Collection of Gastrointestinal Samples from Harvested Ice Seals*

During 2005-2019, samples were collected from ice seals harvested for subsistence purposes between May and September from coastal communities along the coast of the Bering, Chukchi, and Beaufort Seas (Figure 2.1; map created in Google Earth). Information collected included age, sex, length, girth, blubber thickness, and date and location of harvest. General

health assessments for body condition and signs of neurotoxicity were noted by samplers and harvesters. Locations in the Bering Strait and southward were considered to be in the Bering Sea, locations north of the Bering Strait and south of Utqiaġvik were considered to be in the Chukchi Sea, and Utqiaġvik was considered to be in the Beaufort Sea (Logerwell et al., 2011, 2018; Moore & Stabino, 2015; Woodgate et al., 2015).

In the field, whole stomachs were collected in Ziploc® bags and shipped frozen to laboratories where they were stored at -20ºC until they were subsampled. In the laboratory, stomachs were thawed, and 5 ml of semi-liquid content was removed and placed in centrifuge tubes with screw caps before being refrozen. Samples removed from stomachs will hereafter be referred to as "stomach contents." Samples were also collected from the rectum during routine postmortem examination as part of the North Slope Borough Department of Wildlife Management ice seal health monitoring program in Utqiaġvik, Alaska. These samples were stored in 55 cc centrifuge tubes with screw caps and frozen at -20ºC. Samples removed from the rectum will hereafter be referred to as "colon contents." All samples were shipped to the Northwest Fisheries Science Center's Wildlife Algal-Toxin Research and Response Network (WARRN-West) laboratory (NOAA Fisheries, Seattle, Washington, U.S.) for algal toxin testing.

*2.3.2. Quantification of Domoic Acid (DA) and Saxitoxin (STX)*

Toxins were extracted from stomach and colon contents via standard procedures using a 1:3 volume:volume ratio of sample to extraction solvent (Lefebvre et al., 2016). Extraction solvent was 50% methanol for all DA samples, and for 591 STX samples; extraction solvent was 80% ethanol for all other STX samples. Differences in STX concentrations quantified from 50% methanol and 80% ethanol extractions were not found to be statistically significant in n=8 marine

mammal GI samples and are therefore not expected to influence trend analyses (data not shown). Final extracts were further diluted 50-fold for stomach contents and 100-fold for colon contents in dilution buffer prior to DA quantification and 50-fold for both stomach contents and colon contents in dilution buffer prior to STX quantification (Lefebvre et al., 2016). These minimum dilutions were chosen to eliminate matrix effects (Frame & Lefebvre, 2013). Samples and solvent were mixed for one minute, homogenized for 60 s (Omni ES homogenizer), and centrifuged for 20 min at 3,100 rcf (max) at 4ºC (Sorvall RC 5C Plus centrifuge). Finally, supernatant was filtered through a spin filter (Millipore Ultra-Free MC-GV centrifugal filters) spun at 13,870 rcf (max) for 3 min in a desktop centrifuge (Fisher Scientific accuSpin Micro 17). All extracts thus obtained were stored at 4ºC prior to analysis. Concentrations of DA and STX equivalents in nanogram/g (ng/g) were quantified in extracts using commercially available enzyme-linked immunosorbent assay (ELISA) kits for DA (Biosense®) and for STX equivalents (Abraxis®) as per kit instructions. Detection limits for DA in sample material were 4 ng/g for colon contents and 2 ng/g for stomach content. The detection limit for STX in all sample material was 3 ng/g.

It must be noted that the Abraxis STX ELISA kit was specifically designed to detect STX and has limited cross-reactivity with other PST congeners (as listed in the Abraxis product documents). As such, STX concentrations reported here underestimate total potential PST presence. In the absence of data regarding the PST congener profiles in ice seal GI contents, it is difficult to estimate the magnitude of this underestimation. Future studies will include HPLC analyses to characterize the suite of PSTs present in marine mammal tissues as part of our continued research on the trophic transfer of algal toxins in Arctic and subarctic food webs and will be useful for better total PST exposure estimates.

*2.3.3. Analysis of Trends*

Temporal trends in each HAB toxin during 2012-2019 were assessed for bearded seals only and the Bering and Chukchi Seas only, due to sample size limitations for the other three ice seal species and the Beaufort Sea. For consistency, only samples from stomach contents were analyzed for trends. Furthermore, samples were restricted to those collected from May to September, when toxins are expected to be present. First, we examined trends in the prevalence or probability of detection for each HAB toxin. We modeled the probability of occurrence for each toxin using logistic regression. Detections were coded as having a value of 1 and nondetections were coded as having a value of 0. Second, given that a toxin was detected, we examined the trends in the concentration of each toxin using simple linear regression. All analyses were performed using the statistical program R (R Core Team 2018).

## 2.4. Results

Samples were analyzed for the HAB toxins DA and STX from 998 ice seals representing four seal species. Sample collection locations in the Bering, Chukchi, and Beaufort Seas are shown in Figure 2.1. Sex ratios for all species sampled were approximately 1:1, and all age classes (pup, subadult, and adult) were represented for each species.

*2.4.1. Toxin Prevalence and Maximum Concentrations in Ice Seals*

Both DA and STX were detected in all regions sampled (Bering, Chukchi, and Beaufort Seas). Bearded seals had the highest prevalence of DA (46%), followed by ringed (21%), spotted (5%), and ribbon seals (4%) (Table 2.1). Although bearded seals had the highest DA prevalence, ringed seals had the highest DA concentration recorded (1,740 ng DA/g) followed by bearded

seals (1,353 ng DA/g) (Table 2.1). Maximum DA concentrations in spotted and ribbon seals were two orders of magnitude lower at 90 and 33 ng DA/g, respectively. Bearded seals also had the highest prevalence of STX (24%) followed closely by ringed seals (18%). Saxitoxin was only detected in 4% of spotted seals and was not detected in any of the ribbon seals sampled (Table 2.1). Bearded seals had the highest STX concentration (464 ng STX equivalents/g) followed by ringed (180 ng STX equivalents/g) and spotted seals (66 ng STX equivalents/g). Prevalence of co-occurrence (detectable levels of both DA and STX in the same individual) were highest in bearded (17%) and ringed seals (12%) (Table 2.1).

### *2.4.2. Temporal Trends of Toxin Prevalence in Bearded Seals*

The large number of stomach-content samples and the greater geographic span of collection locations for bearded seals allowed for the use of logistic regression to test for temporal trends in toxin prevalence in the Bering and Chukchi seas (Table 2.2). The temporal trend for increasing DA in the Bering Sea was the only significant trend (Figure 2.2; Table 2.2, $p=0.004$). The logistic regression model estimates for the probability of DA presence in 2012 and 2019 were 5% [0%-22%] and 94% [63%-99%], respectively (Table 2.2 and Figure 2.2a). The empirical proportions of DA presence were 0% in 2012 and 100% in 2019 (Figure 2.2a), providing evidence that the regression model accurately describes the trend. No significant trends in the prevalence of STX were observed over the surveyed period (Figure 2.2).

### *2.4.3. Comparison of Toxin Concentrations in Stomach and Colon Content Samples*

To determine if DA and STX concentrations were consistent throughout the GI tract, we compared samples from the same individual at two GI tract locations (stomach and colon) in a

subset of bearded (*N*=10) and ringed (*N*=5) seals. Domoic acid concentrations were higher in colon content samples compared to corresponding stomach content samples in 9 of 10 bearded seals and 5 of 5 ringed seals (Table 2.3). In one bearded seal and two ringed seals, stomach content samples were below detection limits (BDL) for DA, but colon content ranged from 12 to 1,293 ng/g (Table 2.3). The findings for STX concentrations were even more dramatic. Saxitoxin was BDL in stomach content samples from all 15 seals sampled, however, 8 of 10 bearded seals had detectable concentrations in colon content, as did 4 of 5 ringed seals (Table 2.3).

## 2.5. Discussion

Results from this study confirm previous findings that ice seals are regularly exposed to DA and STX in the Bering, Chukchi, and Beaufort Seas (Lefebvre et al., 2016) (Figure 2.2 and Table 2.1). The maximum DA concentration reported here (1,740 ng DA/g in ringed seal feces) is an order of magnitude higher than the maximum concentration of DA previously reported (127 ng DA/g in ringed seal feces) (Lefebvre et al., 2016). The maximum STX concentration reported here (464 ng STX equivalents/g in bearded seal feces) was also higher than the maximum STX concentration previously reported (172 ng STX equivalents/g in ringed seal feces). However, these new maximum values are still well below the seafood safety regulatory limits for humans for both toxins (Table 2.1).

### *2.5.1. Diet and Algal Toxin Prevalence in Ice Seals*

Algal toxin accumulation and prevalence in ice seals occurs through diet. Bearded seals, primarily benthic foragers (Table 2.4), had the highest prevalence of both DA (46%) and STX

(24%) of the four species examined (Table 2.1). Ringed seals, primarily pelagic fish and invertebrate consumers (Table 2.4), had the second highest prevalence of DA (21%) and STX (18%; Table 2.1). Toxin prevalence was lower in the spotted and ribbon seal species, for which pelagic fish are a large part of the diet (5% and 4% for DA and STX in spotted seals, respectively and 4% and 0% for DA and STX in ribbon seals, respectively; Tables 4 and 1). In general, filter-feeding species (benthic and pelagic) accumulate higher concentrations of algal toxins than particulate feeding species due to the direct consumption of algae (Lefebvre, Silver, et al., 2002). A study comparing DA levels in anchovies and sardines collected simultaneously during a toxic *Pseudo-nitzschia* bloom in Monterey, California revealed that anchovies had significantly higher toxin levels than sardines (Lefebvre, Silver, et al., 2002). Although both anchovies and sardines are able to feed on phytoplankton and zooplankton via filter-feeding or particulate/selective feeding modes (Loukashkin, 1970; Radovich, 1952), comparative mouth morphology and feeding behavior suggests that anchovies feed more generally on diatoms, whereas sardines likely target zooplankton, thereby accumulating *Pseudo-nitzschia* secondarily or in lower quantities (Lefebvre, Silver, et al., 2002). Additionally, during toxic *Alexandrium* blooms, benthic shellfish can accumulate high concentrations of STX via both direct consumption of vegetative algal cells and via consumption of benthic cysts of *Alexandrium spp.* from disturbed sediments, allowing for exposure to occur even in the absence of vegetative blooms in surface waters (Persson et al., 2006). Abundant *Alexandrium* cyst beds are present in the sediments of the Chukchi Sea and the eastern Bering Sea (Natsuike et al., 2013). This is consistent with the higher toxin levels and prevalence observed here in bearded seals that primarily consume benthic prey (e.g., flatfish, sculpins, shrimp, crab, gastropods, and clams) and ringed seals that consume filter-feeding invertebrates and planktivorous fish, compared to spotted and ribbon seals that

primarily feed on particulate-consuming pelagic fish (Table 2.4). In a previous study, Pacific walruses (*Odobenus rosmarus divergens*), the most benthic-dependent feeding pinnipeds in the Bering and Chukchi Seas, had the highest toxin concentrations and prevalence for both DA and STX, further suggesting that benthic prey may be the most significant route for exposure (Lefebvre et al., 2016). The fact that planktivorous-fish-consuming ringed seals had the maximum concentrations of both DA and STX reported in previous studies and the maximum STX concentration reported in this study provides further evidence that planktivorous fish are potent vectors of algal toxins.

### *2.5.2. Comparison of Toxin Concentrations in Stomach versus Colon Contents*

Colon content samples consistently had higher toxin levels than corresponding stomach content samples for both DA and STX (Table 2.3). Multiple factors may influence this distribution pattern, including less water content, potential absorption and reabsorption patterns, and that colon content represents more than one stomach's worth of digested material. Regardless, sampling colon contents enhances the ability to detect toxins and is preferable for monitoring toxin prevalence in marine mammals. These results suggest that our previous analyses (Lefebvre et al., 2016) greatly underestimated the prevalence of DA and STX in seals and other marine mammals where stomach content was analyzed. Future monitoring efforts should collect and analyze colon content samples for better estimates of prevalence and concentration even though results will not be directly comparable to past stomach content analysis.

*2.5.3. Temporal Trends of Toxin Prevalence in Bearded Seals*

The significant temporal trend for DA prevalence in bearded seals from 2012-2019 reported above in the Bering Sea (Figure 2.2a) is consistent with a northward expansion of warmer ocean conditions that are favorable for *Pseudo-nitzschia* growth (D. M. Anderson et al., 2018; McCabe et al., 2016). In 2015, a strong link was made between anomalously warm ocean conditions along the U.S. West Coast and Canada, and the development of the largest DA-producing *Pseudo-nitzschia* bloom ever recorded. During this coast-wide bloom, *Pseudo-nitzschia australis* thrived north of its typical range in the warm water that spanned the northeast Pacific (McCabe et al., 2016). Unprecedented levels of DA were found in the northeast Pacific Ocean food web causing coast-wide closures of commercial and recreational fisheries for clams, mussels, Dungeness crab, rock crab, anchovy, and sardine from May to November (McCabe et al., 2016). Unfortunately, concurrent phytoplankton samples were not obtained in the Gulf of Alaska or the Bering Sea, however warmer ocean conditions were also reported in those regions (McCabe et al., 2016). In fact, sea surface temperature data from the Bering Sea show a significant warming trend of $0.22 \pm 0.10^{\circ}C$ $decade^{-1}$ during 1966-2018 (Danielson et al., 2020). Although increasing DA was not observed in bearded seals harvested farther north in the Chukchi Sea, continued northern expansion and increases in *Pseudo-nitzschia* may eventually reach the Chukchi Sea. Additionally, changes in ice seal behavior and regional feeding patterns in response to changing ocean conditions may influence toxin prevalence in the future.

*2.5.4. Exposure Risks for Ice Seals*

Official regulatory limits are 20 µg DA/g (equivalent to 20,000 ng DA/g) shellfish and 80 µg STX equivalents/100 g (equivalent to 800 ng/g) shellfish (Table 2.1) (Wekell et al., 2004).

Regulatory limits were established in seafood for the protection of human health to prevent Amnesic Shellfish Poisoning and Paralytic Shellfish Poisoning from DA and STX, respectively (Wekell et al., 2004). All values reported here were below the seafood safety regulatory limits for both toxins (Table 2.1). Although the concentrations in prey that would be toxic to marine mammals are unknown, regulatory limits can be used as estimates for concentrations in prey that could be harmful to mammalian species.

While some values reported here fall within the range of toxin concentrations quantified in fecal and GI samples from stranded California sea lions diagnosed with acute DA toxicosis (Lefebvre et al., 2016), those levels in sea lions were highly variable (i.e., ranging from 0.001 µg/g to well above seafood safety regulatory limits of >20,000 ng/g; Figure 2 in Lefebvre et al., 2016) and are not a reliable proxy for actual doses of toxin consumed. Consequently, secondary signs of excitotoxicity such as seizures, ataxia, and head weaving are necessary for a positive clinical diagnosis of DA poisoning in marine mammals (Scholin et al., 2000). No clinical signs of DA-induced excitotoxicity or STX-induced paralysis were reported for these seals by the hunters who harvested them. This suggests that algal toxins may not yet be a significant health threat to ice seals, but raises valid concerns about future exposure risks with continued ocean warming as a result of continuing sea ice loss. Because warmer ocean temperatures foster increased harmful algal growth, and Arctic and subarctic regions are undergoing rapid rates of ocean warming, concern for increasing impacts of harmful algal toxins on important marine resources is high (D. M. Anderson et al., 2018). Such impacts are of particular concern for communities where there is a substantial reliance on marine mammals as a food resource (D. M. Anderson et al., 2018; Braund & Associates, 2018; Garlich-Miller & Burn, 1999; MacCracken et al., 2017; Nelson et al., 2019).

## 2.6. Summary

Ice seals (i.e., bearded, ringed, spotted, and ribbon seals) are regularly exposed to both DA and STX in the Bering, Chukchi, and Beaufort Seas. Colon content samples are more sensitive indicators for DA and STX than stomach content samples and should be used in future monitoring efforts. Nonetheless, stomach content analyses in bearded seals were sufficient to identify a significant increase in DA prevalence from 0% in 2012 to 100% in 2019 in the Bering Sea, consistent with warming ocean conditions fostering a northward expansion and increase of *Pseudo-nitzschia* spp.. Differences found in toxin prevalence and concentration among ice seal species are most likely due to diet differences, with filter feeding benthic prey and planktivorous fish likely presenting the greatest exposure risks for ice seals. Observable health impacts for the harvested seals sampled in this study were not reported by hunters. However, consequences of chronic low-level exposure are of concern, as is the possibility that toxin concentrations may increase to harmful levels as Alaskan waters continue to respond to the continuing reduction in seasonal sea ice coverage. Ice seals in general, and bearded seals in particular, can be valuable sentinels for changes in DA and STX prevalence in Pacific Arctic and subarctic marine ecosystems.

## 2.7. Conflicts of Interest

The authors declare that there are no conflicts of interest.

## 2.8. Acknowledgements

This project was made possible by the willingness of hunters to contribute samples from their harvest, by the support of their communities, local governments, and Tribal Councils, and

by the Ice Seal Committee. We would like to express our gratitude to the communities of Gambell, Hooper Bay, Kotzebue, Little Diomede, Nome, Point Hope, Point Lay, Safety Sound, Savoonga, Shishmaref, Tununak, Utqiaġvik, and Wainwright. Without their contribution this study would have not been possible. We thank Mark Nelson, Louise Biderman, Ryan Adam, and college interns from the Alaska Department of Fish and Game (ADF&G) and Cyd Hans, Rita Acker, Olive Kanayurak, and Frances Olemaun from the North Slope Borough Department of Wildlife Management (NSB DWM) for sample collection and processing. John Citta of ADF&G provided guidance regarding statistical analysis. We also thank Don Anderson of Woods Hole Oceanographic Institution for help in editing the manuscript.

**2.9. Funding**

This paper is a result of research funded by the National Oceanic and Atmospheric Administration (NOAA) National Centers for Coastal Ocean Science Competitive Research Program under award number NA20NOS4780195 to the Northwest Fisheries Science Center (NWFSC to KAL) and Woods Hole Oceanographic Institution (to Don Anderson). This is ECOHAB publication number ECO973. Funding for this project was also provided by NOAA's Office of Protected Resources Species of Concern grant (SOC Office of Protective Resources 2009SOC-ice seals to KAL and EF), by the Marine Mammal Commission, by NOAA's National Marine Fisheries Service Projects NA05NMF4391187, NA08NMF4390544, NA11NMF4390200, NA16NMF4720079, and NA16NMF4390029, by NOAA/NWFSC/Wildlife Algal Toxins Research and Response Network (WARRN-West), by a substantial grant from the Coastal Impact Assistance Program (F12AF01265), by qualified outer continental shelf oil and gas revenues, by the NSB DWM, by the National Institutes of Health

(NIH) R01s ES021930 and ES030319 (to DJM and KAL), and by the National Science Foundation (NSF) R01s OCE-1314088 and OCE-1839041 (to DJM and KAL). This work was supported in part by the UW NIEHS sponsored Environmental Pathology/Toxicology Training Program (EP/T) Training Grant (NIEHS T32ES007032). All sample collection from subsistence harvested ice seals was performed under the following permits: National Marine Fisheries Service (NMFS) research permit numbers 358-1787, 15324, and 20466, issued to ADF&G, and 814-1899-00; 814-1899-01; 814-1899-02; 814-1899-03; 814-1899-04; 17350-00; 17350-01, 17350-02, and 21386 issued to NSB DWM.

## 2.10. References

Anderson, C. R., Sapiano, M. R. P., Prasad, M. B. K., Long, W., Tango, P. J., Brown, C. W., & Murtugudde, R. (2010). Predicting potentially toxigenic Pseudo-nitzschia blooms in the Chesapeake Bay. *Journal of Marine Systems*, *83*(3–4), 127–140. https://doi.org/10.1016/j.jmarsys.2010.04.003

Anderson, D. M., Hoagland, P., Kaoru, Y., & White, A. W. (2000). *Estimated Annual Economic Impacts from Harmful Algal Blooms (HABs) in the United States*. (WHOI-2000-11). Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts.

Anderson, D. M., Richlen, M. L., & Lefebvre, K. A. (2018). Harmful Algal Blooms in the Arctic. In E. Osborne, J. Richter-Menge, & M. Jeffries (Eds.), *Arctic Report Card 2018* (pp. 81–87).

Antonelis, G. A., Melin, S. R., & Bukhtiyarov, Y. A. (1994). Early Spring Feeding Habits of Bearded Seals (Erignathus barbatus) in the Central Bering Sea, 1981. *Arctic*, *47*(1), 74–79.

Bargu, S., Silver, M., Goldstein, T., Roberts, K., & Gulland, F. (2010). Complexity of domoic acid-related sea lion strandings in Monterey Bay, California: foraging patterns, climate events, and toxic blooms. *Marine Ecology Progress Series*, *418*, 213–222. https://doi.org/10.3354/meps08816

Bates, N. R., Garley, R., Frey, K. E., Shake, K. L., & Mathis, J. T. (2014). Sea-ice melt CO2-carbonate chemistry in the western Arctic Ocean: Meltwater contributions to air-sea CO2 gas exchange, mixed-layer properties and rates of net community production under sea ice. *Biogeosciences*, *11*(23), 6769–6789. https://doi.org/10.5194/bg-11-6769-2014

Bates, S. S. (2000). Domoic-acid-producing diatoms: Another genus added! *Journal of Phycology*, *36*(6), 978–983. https://doi.org/10.1046/j.1529-8817.2000.03661.x

Bates, S. S., & Trainer, V. L. (2006). The Ecology of Harmful Diatoms. In E. Graneli & J. T. Turner (Eds.), *Ecology of Harmful Algae* (pp. 81–93). Springer. https://doi.org/10.1007/978-3-540-32210-8_7

Berman, F. W., & Murray, T. F. (2002). Domoic Acid Neurotoxicity in Cultured Cerebellar Granule Neurons Is Mediated Predominantly by NMDA Receptors that Are Activated as a Consequence of Excitatory Amino Acid Release. *Journal of Neurochemistry*, *69*(2), 693–703. https://doi.org/10.1046/j.1471-4159.1997.69020693.x

Bintanja, R., & van der Linden, E. C. (2013). The changing seasonal climate in the Arctic. *Scientific Reports*, *3*, 1556-undefined. https://doi.org/10.1038/srep01556

Braund, S. R & Associates. (2018) Description of Alaskan Eskimo bowhead whale subsistence sharing practices. Final report submitted to the Alaska Eskimo Whaling Commission, May 2018.

Bukhtiyarov, Y. A., Frost, K. J., & Lowry, L. E. (1984). Phoca largha, in the Bering Sea in Spring. In F. H. Fay & G. A. Fedoseev (Eds.), *Soviet-America cooperative research on marine mammals, Pinnipeds* (Vol. 1, pp. 55–59). National Marine Fisheries Service, National Oceanic and Atmospheric Administration, U.S. Department of Commerce.

Capotondi, A., Alexander, M. A., Bond, N. A., Curchitser, E. N., & Scott, J. D. (2012). Enhanced upper ocean stratification with climate change in the CMIP3 models. *Journal of Geophysical Research: Oceans*, *117*(4). https://doi.org/10.1029/2011JC007409

Cembella, A., & Desbiens, M. (1994). Fate of paralytic shellfish toxins in the American lobster Homarus americanus. *Journal of Shellfish Research*, *13*, 302.

Cook, P. F., Reichmuth, C., Rouse, A. A., Libby, L. A., Dennison, S. E., Carmichael, O. T., Kruse-Elliott, K. T., Bloom, J., Singh, B., Fravel, V. A., Barbosa, L., Stuppino, J. J., van Bonn, W. G., Gulland, F., & Ranganath, C. (2015). Algal toxin impairs sea lion memory and hippocampal connectivity, with implications for strandings. *Science*, *350*(6267), 1545–1547. https://doi.org/10.1126/science.aac5675

Crawford, J. A., Quakenbush, L. T., & Citta, J. J. (2015). A comparison of ringed and bearded seal diet, condition and productivity between historical (1975-1984) and recent (2003-2012) periods in the Alaskan Bering and Chukchi seas. *Progress in Oceanography*, *136*, 133–150. https://doi.org/10.1016/j.pocean.2015.05.011

Danielson, S. L., Ahkinga, O., Ashjian, C., Basyuk, E., Cooper, L. W., Eisner, L., Farley, E., Iken, K. B., Grebmeier, J. M., Juranek, L., Khen, G., Jayne, S. R., Kikuchi, T., Ladd, C., Lu, K., McCabe, R. M., Moore, G. W. K., Nishino, S., Ozenna, F., … Weingartner, T. J. (2020). Manifestation and consequences of warming and altered heat fluxes over the Bering and Chukchi Sea continental shelves. *Deep-Sea Research Part II: Topical Studies in Oceanography*, *177*, 104781. https://doi.org/10.1016/j.dsr2.2020.104781

DeGange, A. R., & Vacca, M. M. (1989). Sea Otter Mortality at Kodiak Island, Alaska, during Summer 1987. *Journal of Mammalogy*, *70*(4), 836–838. https://doi.org/10.2307/1381723

Dehn, L.-A., Sheffield, G. G., Follmann, E. H., Duffy, L. K., Thomas, D. L., & O'Hara, T. M. (2007). Feeding ecology of phocid seals and some walrus in the Alaskan and Canadian Arctic as determined by stomach contents and stable isotope analysis. *Polar Biology*, *30*, 167–181. https://doi.org/10.1007/s00300-006-0171-0

Etheridge, S. M. (2010). Paralytic shellfish poisoning: Seafood safety and human health perspectives. *Toxicon*, *56*(2), 108–122. https://doi.org/10.1016/j.toxicon.2009.12.013

Flewelling, L. J., Naar, J. P., Abbott, J. P., Baden, D. G., Barros, N. B., Bossart, G. D., Bottein, M. Y. D., Hammond, D. G., Haubold, E. M., Heil, C. A., Henry, M. S., Jacocks, H. M., Leighfield, T. A., Pierce, R. H., Pitchford, T. D., Rommel, S. A., Scott, P. S., Steidinger, K. A., Truby, E. W., … Landsberg, J. H. (2005). Brevetoxicosis: Red tides and marine mammal mortalities. *Nature*, *435*(7043), 755–756. https://doi.org/10.1038/nature435755a

Frame, E., & Lefebvre, K. A. (2013). *ELISA Methods for Domoic Acid Quantification in Multiple Marine Mammal Species and Sample Matrices* (NOAA Technical Memorandum NMFS-NWFSC-122). U.S. Department of Commerce.

Fritz, L., Quilliam, M. A., Wright, J. L. C., Beale, A. M., & Work, T. M. (1992). An outbreak of domoic acid poisoning attributed to the pennate diatom Pseudonitzschia australis. *Journal of Phycology*, *28*(4), 439–442. https://doi.org/10.1111/j.0022-3646.1992.00439.x

Frost, K. J., & Lowry, L. F. (1980). Feeding of ribbon seals (Phoca fasciata) in the Bering Sea in spring. *Canadian Journal of Zoology*, *58*(9), 1601–1607. https://doi.org/10.1139/z80-219

Garlich-Miller, J., & Burn, D. (1999). Estimating the harvest of Pacific walrus, Odobenus rosmarus divergens, in Alaska. *Fishery Bulletin*, *97*(4), 1043–1046.

Geraci, J. R., Anderson, D. M., Timperi, R. J., St. Aubin, D. J., Early, G. A., Prescott, J. H., & Mayo, C. A. (1989). Humpback Whales (Megaptera novaeangliae) Fatally Poisoned by Dinoflagellate Toxin. *Canadian Journal of Fisheries and Aquatic Sciences*, *46*(11), 1895–1898. https://doi.org/10.1139/f89-238

Goldstein, T., Mazet, J. A. K., Zabka, T. S., Langlois, G., Colegrove, K. M., Silver, M., Bargu, S., van Dolah, F., Leighfield, T., Conrad, P. A., Barakos, J., Williams, D. C., Dennison, S., Haulena, M., & Gulland, F. M. D. (2008). Novel symptomatology and changing epidemiology of domoic acid toxicosis in California sea lions (Zalophus californianus): An increasing risk to marine mammal health. *Proceedings of the Royal Society B: Biological Sciences*, *275*(1632), 267–276. https://doi.org/10.1098/rspb.2007.1221

Gulland, F. (2000). *Domoic acid toxicity in California sea lions (Zalophus Californianus) stranded along the central California coast, May - October 1998* (NOAA Technical Memorandum NMFS-OPR-17*)*. National Marine Fisheries Service, National Oceanic and Atmospheric Administration, U.S. Department of Commerce.

Gulland, F., & Hall, A. J. (2007). Is marine mammal health deteriorating? Trends in the global reporting of marine mammal disease. *EcoHealth*, *4*(2), 135–150. https://doi.org/10.1007/s10393-007-0097-1

Gulland, F., Pérez-Cortés, H., Urbán, J., Rojas-Bracho, L., Ylitalo, G., Weir, J., Norman, S. A., Muto, M. M., Rugh, D. J., Kreuder, C., & Rowles, T. (2005). *Eastern North Pacific Gray Whale (Eschrichtius robustus) Unusual Mortality Event, 1999-2000* (NOAA Technical Memorandum NMFS-AFSC-150). National Marine Fisheries Service, National Oceanic and Atmospheric Administration, U.S. Department of Commerce.

Hu, A., Meehl, G. A., Han, W., Timmermann, A., Otto-Bliesner, B., Liu, Z., Washington, W. M., Large, W., Abe-Ouchi, A., Kimoto, M., Lambeck, K., & Wu, B. (2012). Role of the Bering Strait on the hysteresis of the ocean conveyor belt circulation and glacial climate stability. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(17), 6417–6422. https://doi.org/10.1073/pnas.1116014109

Johannessen, O. M., Bengtsson, L., Miles, M. W., Kuzmina, S. I., Semenov, V. A., Alekseev, G. v., Nagurnyi, A. P., Zakharov, V. F., Bobylev, L. P., Pettersson, L. H., Hasselmann, K., & Cattle, H. P. (2004). Arctic climate change: observed and modelled temperature and sea-

ice variability. *Tellus A: Dynamic Meteorology and Oceanography*, *56*(4), 328–341. https://doi.org/10.3402/tellusa.v56i4.14418

Johnson, M. L., Fiscus, C. H., Ostenson, B. T., & Barbour, M. L. (1966). Marine mammals. In N. J. Wilimovsky & J. N. Wolfe (Eds.), *Environment of the Cape Thompson region, Alaska* (pp. 877–924). US Atomic Energy Commission.

Kvitek, R., Goldberg, J., Smith, G., Doucette, G., & Silver, M. (2008). Domoic acid contamination within eight representative species from the benthic food web of Monterey Bay, California, USA. *Marine Ecology Progress Series*, *367*, 35–47. https://doi.org/10.3354/meps07569

Laidre, K. L., Stern, H., Kovacs, K. M., Lowry, L., Moore, S. E., Regehr, E. v., Ferguson, S. H., Wiig, Ø., Boveng, P., Angliss, R. P., Born, E. W., Litovka, D., Quakenbush, L., Lydersen, C., Vongraven, D., & Ugarte, F. (2015). Arctic marine mammal population status, sea ice habitat loss, and conservation recommendations for the 21st century. *Conservation Biology*, *29*(3), 724–737. https://doi.org/10.1111/cobi.12474

Landsberg, J., Lefebvre, K. A., & Flewelling, L. (2014). Effects of Toxic Microalgae on Marine Organisms. In *Toxins and Biologically Active Compounds from Microalgae, Volume 2* (pp. 379–449). CRC Press. https://doi.org/10.1201/b16806-17

Lefebvre, K. A., Bargu, S., Kieckhefer, T., & Silver, M. W. (2002). From sanddabs to blue whales: The pervasiveness of domoic acid. *Toxicon*, *40*(7), 971–977. https://doi.org/10.1016/S0041-0101(02)00093-4

Lefebvre, K. A., Powell, C. L., Busman, M., Doucette, G. J., Moeller, P. D. R., Silver, J. B., Miller, P. E., Hughes, M. P., Singaram, S., Silver, M. W., & Tjeerdema, R. S. (1999). Detection of domoic acid in northern anchovies and California sea lions associated with an unusual mortality event. *Natural Toxins*, *7*(3), 85–92. https://doi.org/10.1002/(SICI)1522-7189(199905/06)7:3<85::AID-NT39>3.0.CO;2-Q

Lefebvre, K. A., Quakenbush, L., Frame, E., Huntington, K. B., Sheffield, G., Stimmelmayr, R., Bryan, A., Kendrick, P., Ziel, H., Goldstein, T., Snyder, J. A., Gelatt, T., Gulland, F., Dickerson, B., & Gill, V. (2016). Prevalence of algal toxins in Alaskan marine mammals foraging in a changing arctic and subarctic environment. *Harmful Algae*, *55*, 13–24. https://doi.org/10.1016/j.hal.2016.01.007

Lefebvre, K. A., Robertson, A., Frame, E. R., Colegrove, K. M., Nance, S., Baugh, K. A., Wiedenhoft, H., & Gulland, F. (2010). Clinical signs and histopathology associated with domoic acid poisoning in northern fur seals (Callorhinus ursinus) and comparison of toxin detection methods. *Harmful Algae*, *9*(4), 374–383. https://doi.org/10.1016/j.hal.2010.01.007

Lefebvre, K. A., Silver, M. W., Coale, S. L., & Tjeerdema, R. S. (2002). Domoic acid in planktivorous fish in relation to toxic Pseudo-nitzschia cell densities. *Marine Biology*, *140*(3), 625–631. https://doi.org/10.1007/s00227-001-0713-5

Logerwell, E., Rand, K., Danielson, S., & Sousa, L. (2018). Environmental drivers of benthic fish distribution in and around Barrow Canyon in the northeastern Chukchi Sea and western Beaufort Sea. *Deep-Sea Research Part II, 152*, 170-181.

Logerwell, E., Rand, K., & Weingartner, T. J. (2011). Oceanographic characteristics of the habitat of benthic fish and invertebrates in the Beaufort Sea. *Polar Biology*, *34,* 1783-1796.

Loukashkin, A. S. (1970). On the diet and feeding behavior of the northern Anchovy Engraulis mordax (Girard). *Proceedings of the California Academy of Sciences, 4th Series*, *37*, 419–458.

Lowry, L. F., Frost, K. J., & Burns, J. J. (1980a). Feeding of Bearded Seals in the Bering and Chukchi Seas and Trophic Interaction with Pacific Walruses. *Arctic*, *33*, 330–342. https://doi.org/10.14430/arctic2566

Lowry, L. F., Frost, K. J., & Burns, J. J. (1980b). Variability in the Diet of Ringed Seals, Phoca hispida. *Canadian Journal of Fisheries Ad Aquatic Sciences*, *37*, 2254–2261. https://doi.org/10.1139/f80-270

Lowry, L., & Frost, K. (1981). Feeding and trophic relationships of phocid seals and walrus in the eastern Bering Sea. In D. Hood & J. Calder (Eds.), *The eastern Bering Sea shelf: oceanography and resources* (Vol. 2, pp. 813–824). Office of Marine Pollution Assessment, National Oceanic and Atmospheric Administration, U.S. Department of Commerce.

MacCracken, J. G., Beatty, W. S., Garlich-Miller, J. L., Kissling, M. L., & Snyder, J. A. (2017). *Final species status assessment for the Pacific walrus (Odobenus rosmarus divergens), May 2017 (Version 1.0)*. Marine Mammals Management, U.S. Fish and Wildlife Service. https://doi.org/10.13140/RG.2.2.29363.12325

McCabe, R. M., Hickey, B. M., Kudela, R. M., Lefebvre, K. A., Adams, N. G., Bill, B. D., Gulland, F., Thomson, R. E., Cochlan, W. P., & Trainer, V. L. (2016). An unprecedented coastwide toxic algal bloom linked to anomalous ocean conditions. *Geophysical Research Letters*, *43*(19), 10,366-10,376. https://doi.org/10.1002/2016GL070023

McKenzie, C. H., Bates, S. S., Martin, J. L., Haigh, N., Howland, K. L., Lewis, N. I., Locke, A., Peña, A., Poulin, M., Rochon, A., Rourke, W. A., Scarratt, M. G., Starr, M., & Wells, T. (in press). Three decades of Canadian marine harmful algal events: Phytoplankton and phycotoxins of concern to human and ecosystem health. *Harmful Algae*. https://doi.org/10.1016/j.hal.2020.101852

Moore, S. E., & Stabeno, P. J. (2015). Synthesis of Arctic Research (SOAR) in marine ecosystems of the Pacific Arctic. *Progress in Oceanography 136*: 1-11.

Natsuike, M., Nagai, S., Matsuno, K., Saito, R., Tsukazaki, C., Yamaguchi, A., & Imai, I. (2013). Abundance and distribution of toxic Alexandrium tamarense resting cysts in the sediments of the Chukchi Sea and the eastern Bering Sea. *Harmful Algae*, *27*, 52–59. https://doi.org/10.1016/j.hal.2013.04.006

Nelson, M., Quakenbush, L., Taras, B., & Ice Seal, C. (2019). Subsistence harvest of ringed, bearded, spotted, and ribbon seals in Alaska is sustainable. *Endangered Species Research*, *40*, 1–16. https://doi.org/10.3354/esr00973

Peery, M. Z., Beissinger, S. R., Burkett, E., & Newman, S. H. (2006). Local Survival of Marbled Murrelets in Central California: Roles of Oceanographic Processes, Sex, and Radiotagging. *Journal of Wildlife Management*, *70*(1), 78–88. https://doi.org/10.2193/0022-541x(2006)70[78:lsommi]2.0.co;2

Perl, T. M., Bédard, L., Kosatsky, T., Hockin, J. C., Todd, E. C. D., & Remis, R. S. (1990). An Outbreak of Toxic Encephalopathy Caused by Eating Mussels Contaminated with Domoic Acid. *New England Journal of Medicine*, *322*(25), 1775–1780. https://doi.org/10.1056/NEJM199006213222504

Persson, A., Smith, B. C., Wikfors, G. H., & Quilliam, M. (2006). Grazing on toxic Alexandrium fundyense resting cysts and vegetative cells by the eastern oyster (Crassostrea virginica). *Harmful Algae*, *5*(6), 678–684. https://doi.org/10.1016/j.hal.2006.02.004

Pickart, R. S., Schulze, L. M., Moore, G. W. K., Charette, M. A., Arrigo, K. R., van Dijken, G., & Danielson, S. L. (2013). Long-term trends of upwelling and impacts on primary productivity in the Alaskan Beaufort Sea. *Deep-Sea Research Part I: Oceanographic Research Papers*, *79*, 106–121. https://doi.org/10.1016/j.dsr.2013.05.003

Radovich, J. (1952). Food of the Pacific sardine, Sardinops caerulea, from central Baja California and southern California. *California Fish and Game Bulletin*, *3*(48), 575–585.

Scholin, C. A., Gulland, F., Doucette, G. J., Benson, S., Busman, M., Chavez, F. P., Cordaro, J., DeLong, R., de Vogelaere, A., Harvey, J., Haulena, M., Lefebvre, K., Lipscomb, T., Loscutoff, S., Lowenstine, L. J., Marin, R., Miller, P. E., McLellan, W. A., Moeller, P. D. R., … van Dolah, F. M. (2000). Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature*, *403*(6765), 80–84. https://doi.org/10.1038/47481

Shumway, S. E. (1990). A Review of the Effects of Algal Blooms on Shellfish and Aquaculture. *Journal of the World Aquaculture Society*, *21*(2), 65–104. https://doi.org/10.1111/j.1749-7345.1990.tb00529.x

Stevenson, D. E., & Lauth, R. R. (2019). Bottom trawl surveys in the northern Bering Sea indicate recent shifts in the distribution of marine species. *Polar Biology*, *42*(2), 407–421. https://doi.org/10.1007/s00300-018-2431-1

Stone, R. S., Dutton, E. G., Harris, J. M., & Longenecker, D. (2002). Earlier spring snowmelt in northern Alaska as an indicator of climate change. *Journal of Geophysical Research: Atmospheres*, *107*(9–10), ACL 10-1. https://doi.org/10.1029/2000jd000286

Todd, E. C. D. (1993). Domoic Acid and Amnesic Shellfish Poisoning - A Review. *Journal of Food Protection*, *56*(1), 69–83. https://doi.org/10.4315/0362-028x-56.1.69

Trainer, V. L., Cochlan, W. P., Erickson, A., Bill, B. D., Cox, F. H., Borchert, J. A., & Lefebvre, K. A. (2007). Recent domoic acid closures of shellfish harvest areas in Washington State inland waterways. *Harmful Algae*, *6*(3), 449–459. https://doi.org/10.1016/j.hal.2006.12.001

Tremblay, J.-É., & Gagnon, J. (2009). The effects of irradiance and nutrient supply on the productivity of Arctic waters: a perspective on climate change. In J. C. J. Nihoul & A. G. Kostianoy (Eds.), *Influence of Climate Change on the Changing Arctic and Sub-Arctic Conditions* (pp. 73–93). Springer Netherlands. https://doi.org/10.1007/978-1-4020-9460-6_7

Turner, J., & Overland, J. (2009). Contrasting climate change in the two polar regions. *Polar Research*, *28*(2), 146–164. https://doi.org/10.1111/j.1751-8369.2009.00128.x

U.S. Federal Register. 2012a. *Threatened Status for the Arctic, Okhotsk, and Baltic Subspecies of the Ringed Seal and Endangered Status for the Ladoga Subspecies of the Ringed Seal*. FR 77(249):76706–76738 (28 Dec. 2012). National Oceanic and Atmospheric Administration, U.S. Department of Commerce.

U.S. Federal Register. 2012b. *Threatened Status for the Beringia and Okhotsk Distinct Population Segments of the Erignathus barbatus nauticus Subspecies of the Bearded Seal*. FR 77(249):76740–76768 (28 Dec. 2012). National Oceanic and Atmospheric Administration, U.S. Department of Commerce.

Usup, G., Kulis, D. M., & Anderson, D. M. (1994). Growth and toxin production of the toxic dinoflagellate Pyrodinium bahamense var. compressum in laboratory cultures. *Natural Toxins*, *2*(5), 254–262. https://doi.org/10.1002/nt.2620020503

Wekell, J. C., Hurst, J., & Lefebvre, K. A. (2004). The origin of the regulatory limits for PSP and ASP toxins in shellfish. *Article in Journal of Shellfish Research*, *23*(3), 927–930.

White, A. W. (1980). Recurrence of Kills of Atlantic Herring (Clupea harengus harengus) Caused by Dinoflagellate Toxins Transferred Through Herbivorous Zooplankton. *Canadian Journal of Fisheries and Aquatic Sciences*, *37*(12), 2262–2265. https://doi.org/10.1139/f80-271

White, A. W. (1981). Marine zooplankton can accumulate and retain dinoflagellate toxins and cause fish kills. *Limnology and Oceanography*, *26*(1), 103–109. https://doi.org/10.4319/lo.1981.26.1.0103

Woodgate, R. A., Stafford, K. M., & Prahl, F. G. (2015). A synthesis of year-round interdisciplinary mooring measurements in the bering Strait (1990-2014) and the RUSALCA years (2004-2011). *Oceanography 28(3)* 46-67. http://dx.doi.org/10.5670/oceanog.2015.57

Work, T. M., Barr, B., Beale, A. M., Fritz, L., Quilliam, M. A., & Wright, J. L. C. (1993). Epidemiology of Domoic Acid Poisoning in Brown Pelicans (Pelecanus occidentalis) and Brandt's Cormorants (Phalacrocorax penicillatus) in California. *Journal of Zoo and Wildlife Medicine*, *24*(1), 54–62.

Zhu, Z., Qu, P., Fu, F., Tennenbaum, N., Tatters, A. O., & Hutchins, D. A. (2017). Understanding the blob bloom: Warming increases toxicity and abundance of the harmful bloom diatom Pseudo-nitzschia in California coastal waters. *Harmful Algae*, *67*, 36–43. https://doi.org/10.1016/j.hal.2017.06.004

## 2.11. Tables and Figures

**Table 2.1.** Prevalence of domoic acid (DA) and saxitoxin (STX) in gastrointestinal samples by species. Maximum concentrations (maximum [DA] and [STX]) did not reach regulatory limits for either DA (regulatory limit = 20,000 ng DA/g shellfish*) or STX (regulatory limit = 800 ng STX equivalents/g shellfish*). *n* = number of animals.

* Regulatory limit units have been converted to match those reported in the table below.

| **Common name (Latin name)** | **Collection Years** | ***n* DA positive / *n* DA tested (% DA positive)** | ***n* STX positive/ *n* STX tested (% STX positive)** | ***n* DA & STX positive (% co-occurrence)** | **Max [DA] (ng/g)** | **Max [STX] (ng/g)** |
|---|---|---|---|---|---|---|
| Bearded seal *(Erignathus barbatus)* | 2005-2019 | 157/344 (46%) | 96/404 (24%) | 69 (17%) | 1353 | 464 |
| Ringed seal *(Pusa hispida)* | 2005-2019 | 61/289 (21%) | 47/263 (18%) | 31 (12%) | 1740 | 180 |
| Spotted seal *(Phoca largha)* | 2005-2016 | 14/268 (5%) | 9/257 (4%) | 1 (0%) | 90 | 66 |
| Ribbon seal *(Histriophoca fasciata)* | 2008-2016 | 1/28 (4%) | 0/28 (0%) | 0 (0%) | 33 | 0 |

**Table 2.2.** Proportion of bearded seal stomach content samples collected in the Bering Sea that were found to have domoic acid (DA) by year and fitted logistic regression probabilities by year with 95% confidence intervals (CI). Fewer than 3 samples were collected in 2018 from the Bering Sea, therefore it was excluded from analysis.

| Year | Samples collected | Samples positive for DA | Proportion positive for DA | Logistic regression estimates of DA probability [95% CI] |
|---|---|---|---|---|
| 2012 | 4 | 0 | 0 | 0.05 [0.01, 0.22] |
| 2013 | 7 | 1 | 0.14 | 0.10 [0.03, 0.30] |
| 2014 | 8 | 4 | 0.50 | 0.20 [0.09, 0.40] |
| 2015 | 14 | 2 | 0.14 | 0.37 [0.22, 0.54] |
| 2016 | 6 | 3 | 0.50 | 0.57 [0.36, 0.75] |
| 2017 | 3 | 3 | 1.00 | 0.75 [0.46, 0.91] |
| 2018 | NA | NA | NA | 0.87 [0.55, 0.97] |
| 2019 | 5 | 5 | 1.00 | 0.94 [0.63, 0.99] |

**Table 2.3.** Comparison of toxin concentrations detected in samples from two gastrointestinal tract locations (stomach and colon) collected simultaneously in 15 seals. For each seal, the highest toxin concentration is in bold. BDL = below detection limits.

| Animal ID | Species | DA concentration (ng/g) | | STX concentration (ng/g) | |
|---|---|---|---|---|---|
| | | Stomach Content | Colon Content | Stomach Content | Colon Content |
| 2012BS07 | Bearded seal | 2 | **4** | BDL | **8** |
| 09BS2 | Bearded seal | 10 | **156** | BDL | **10** |
| 09BS20 | Bearded seal | 7 | **23** | BDL | BDL |
| 09BS21 | Bearded seal | BDL | **12** | BDL | **15** |
| 09BS22 | Bearded seal | 138 | **887** | BDL | BDL |
| 09BS3 | Bearded seal | 3 | **7** | BDL | **3** |
| 09BS4 | Bearded seal | 3 | **11** | BDL | **6** |
| 09BS7 | Bearded seal | **5** | BDL | BDL | **8** |
| 09BS8 | Bearded seal | 6 | **136** | BDL | **108** |
| 09BS9 | Bearded seal | 8 | **12** | BDL | **23** |
| 09RS8 | Ringed seal | 7 | **15** | BDL | **180** |
| 2011RS2 | Ringed seal | 6 | **19** | BDL | **29** |
| 2015-RS-10 | Ringed seal | 7 | **113** | BDL | **6** |
| 2015RS12 | Ringed seal | BDL | **142** | BDL | **4** |
| 2015RS13 | Ringed seal | BDL | **1293** | BDL | BDL |

**Table 2.4.** Primary known prey species for bearded, ringed, spotted, and ribbon seals.

| **Species (*Latin name*)** | **Feeding preferences** | **Invertebrate prey** | **Fish prey** | **References** |
|---|---|---|---|---|
| Bearded seals *(Erignathus barbatus)* | Benthic fish and invertebrates | Bivalves<br>Gastropods<br>Cephalopods<br>Isopods<br>Amphipods<br>Shrimps<br>Crabs<br>Echiurids<br>Polychaetes | Pelagic<br>Arctic cod (*Boreogadus saida*)<br>Saffron cod (*Eleginus gracilis*)<br>Benthic<br>Sculpins (Cottidae)<br>Snailfish (Liparidae)<br>Pricklebacks (Stichaeidae)<br>Pacific sand lance (*Ammodytes hexapterus)*<br>Flatfish (Pleuronectidae) | (Antonelis et al., 1994; Crawford et al., 2015; L. F. Lowry et al., 1980a; ADF&G *unpublished data*) |
| Ringed seal (*Pusa hispida*) | Pelagic fish and invertebrates | Mysids<br>Amphipods<br>Shrimp | Pelagic<br>Arctic cod (*Boreogadus saida*)<br>Saffron cod (*Eleginus gracilis*)<br>Walleye pollock (*Gadus chalcogramma*)<br>Rainbow smelt (*Osmerus mordax*)<br>Benthic<br>Sculpins (Cottidae) | (Crawford et al., 2015; Dehn et al., 2007; Johnson et al., 1966; L. F. Lowry et al., 1980b; ADF&G *unpublished data*) |
| Spotted seal (*Phoca largha*) | Pelagic fish | Not a significant dietary component | Pelagic<br>Arctic cod (*Boreogadus saida*)<br>Saffron cod (*Eleginus gracilis*)<br>Pacific herring (*Clupea pallasi*)<br>Capelin (*Mallotus villosus*)<br>Rainbow smelt (*Osmerus mordax*) | (Bukhtiyarov et al., 1984; L. Lowry & Frost, 1981; ADF&G *unpublished data*) |

**Table 2.4.** *continued*

| | | | | |
|---|---|---|---|---|
| Ribbon seals (*Histriophoca fasciata*) | Pelagic fish and invertebrates | Shrimp<br>Octopus | <u>Pelagic</u><br>Arctic cod (*Boreogadus saida*)<br>Saffron cod (*Eleginus gracilis*)<br>Walleye pollock (*Gadus chalcogramma*) | (Dehn et al., 2007; Frost & Lowry, 1980; ADF&G *unpublished data*) |


**Figure 2.1.** Harvest locations (black pins) are shown within circles indicating regional classifications (Bering, Chukchi, and Beaufort Seas). Next to each harvest location, icons represent the number of each seal species that tested positive for DA (yellow), STX (red), and both toxins (orange). Map generated in Google Earth.

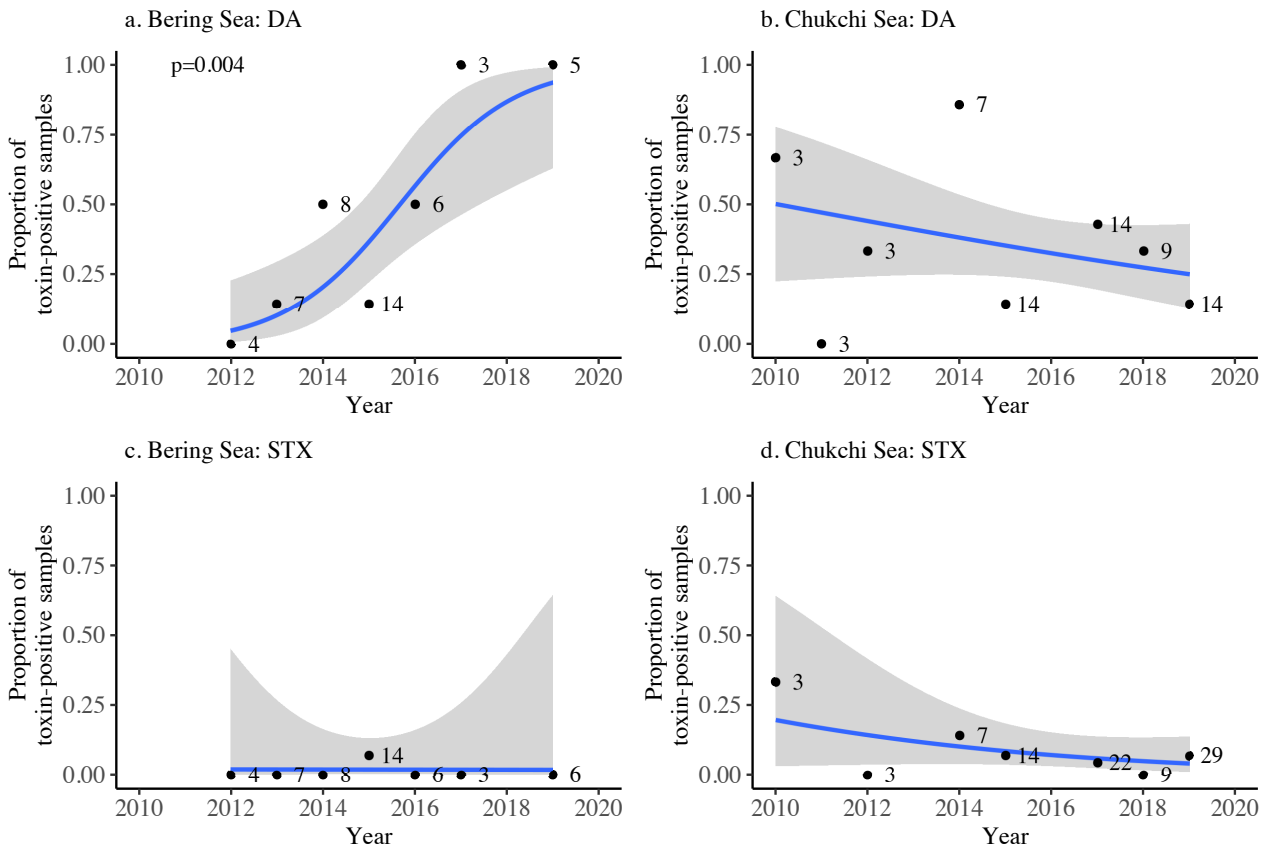


**Figure 2.2.** The proportion of bearded seal stomach content samples with detectable concentrations of domoic acid (DA) (a, b) and saxitoxin (STX) (c, d) from May-September in the Bering (a, c) and Chukchi (b, d) Seas by year. Sample size is listed to the right of each corresponding data point. Lines represent logistic regressions comparing presence/absence of toxin over the years, and shaded areas represent associated 95% confidence intervals. The only significant trend (p=0.004) was in the Bering Sea (a).

**Chapter 3: Age and Sex as Determinants of Acute Domoic Acid Toxicity in a Mouse Model**

*This chapter has been accepted for publication in Toxins. The authors are:*

Alicia M. Hendrix[1], Kathi A. Lefebvre[2], Emily K. Bowers[2], Rudolph Stuppard[3], Thomas M. Burbacher[1] and David J. Marcinek[3]

[1]Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA; aliciah1@uw.edu (A.M.H.), tmb@uw.edu (T.B.)

[2]Environmental Fisheries Science Division, Northwest Fisheries Science Center, National Marine Fisheries Service, National Oceanic and Atmospheric Administration, Seattle, WA; kathi.lefebvre@noaa.gov (K.A.L.), emily.bowers@noaa.gov (E.K.B.)

[3]Departments of Radiology and Laboratory Medicine and Pathology, University of Washington Medical School, Seattle, WA; rudy@uw.edu (R.S.), dmarc@uw.edu (D.J.M.)

### 3.1. Abstract

The excitatory neurotoxin domoic acid (DA) consistently contaminates food webs in coastal regions around the world. Acute exposure to the toxin causes Amnesic Shellfish Poisoning, a potentially lethal syndrome of gastrointestinal and seizure-related outcomes. Both advanced age and male sex have been suggested to contribute to interindividual DA susceptibility. To test this, we administered DA doses between 0.5 and 2.5 mg/kg bw to female and male C57Bl/6 mice at adult (7-9-month-old) and aged (25-28-month-old) life stages and observed seizure-related activity for 90 minutes, at which point we euthanized the mice and collected serum, cortical, and kidney samples. We observed severe clonic-tonic convulsions in some aged individuals, but not in younger adults. We also saw an association between advanced age and the incidence of a moderately severe seizure-related outcome, hindlimb tremors, and between advanced age and overall symptom severity and persistence. Surprisingly, we additionally report that female mice, particularly aged female mice, demonstrated more severe neurotoxic symptoms following acute exposure to DA than males. Both age and sex patterns were reflected in tissue DA concentrations as well: aged mice and females had generally higher concentrations of DA in their tissues at 90 minutes post-exposure. This study contributes to the body of work that can inform intelligent, evidence-based public health protections for communities threatened by more frequent and extensive DA-producing algal blooms.

**Keywords:** domoic acid; aging; seizures; excitotoxicity; amnesic shellfish poisoning

**Key Contribution:** Advanced age is associated with more severe neurotoxicity following exposure to domoic acid doses between 0.5 and 2.5 mg/kg bw in C57Bl/6 mice. Female mice of more advanced age appear to be particularly susceptible.

### 3.2. Introduction

Harmful algal blooms (HABs) are phenomena in which marine or aquatic algal species rapidly produce large amounts of biomass and/or potent toxins, that can adversely impact ecosystem, wildlife, and human health. One such HAB toxin is domoic acid (DA), an excitatory neurotoxin naturally produced by marine algae in the genera *Pseudo-nitzschia* and *Nitzschia* [1–3]. During DA contamination events, filter feeding organisms such as clams and planktivorous fish consume toxic *Pseudo-nitzschia* and can transfer DA to higher trophic levels including humans [4–6]. Acute DA poisoning in human seafood consumers is called Amnesic Shellfish Poisoning (ASP) and was first described following a human poisoning event in 1987 in Prince Edward Island (PEI), Canada. Clinical signs of ASP included gastrointestinal symptoms like vomiting, abdominal cramps and diarrhea, and neurologic symptoms like confusion, disorientation, loss of short-term memory, seizures, and coma [7].

In addition to identifying DA as a seafood toxin and characterizing its associated acute toxicosis, the canonical PEI poisoning event also offered some initial evidence of interindividual susceptibilities to the toxin. Both male sex and increased age (in 10-year increments) were positively correlated with memory loss (estimated odds ratio [OR] of 4.4 and 1.6, respectively)

and hospitalization (estimated OR of 16.9 and 2.3, respectively) following toxin exposure [7]. At the time researchers suggested that age-associated susceptibility might be mediated by age-associated renal function impairment, and that male reluctance to seek medical care might have biased data toward more severe symptoms in men [7]. Since then, follow up studies on age- and sex-associated susceptibility to acute DA poisoning have been very limited [8].

Recent consumption surveys distributed by the Washington Department of Fish and Wildlife suggest that some commercial and recreational razor clam harvesters in the state are regularly exposed to DA at or above the current acute reference dose, i.e., at or above levels deemed safe for human consumption [9]. Further, the surveys reveal that consumers over the age of 60 are commonly represented in this exposed group [9]. Additional 2022 surveys in China estimated higher dietary DA exposures in consumers of more advanced age, though overall estimated DA intake was still quite low [10]. As human exposure to DA is occurring and will likely increase as oceans warm and HAB frequency accordingly increases, it is imperative that we clarify the factors contributing to interindividual DA susceptibilities. This will allow us to create the most appropriate protective and useful public health guidelines. These findings could also be important for marine mammal health as it is well known that California sea lions are frequently exposed to DA as well, and regularly experience DA toxicosis [11]. To address the knowledge gaps in possible age- and sex-associated susceptibilities to DA, we conducted a dose-response study with adult and aged male and female C57Bl/6 mice, assessing gross toxicity and seizure-related behavior.

## 3.3. Results

### *3.3.1. Associations of Dose, Sex, and Age with Clonic-Tonic Convulsion Risk*

One hundred mice were monitored for 90 minutes following DA injection. If during that time a mouse experienced clonic-tonic convulsions (CTCs) lasting longer than 30 seconds, the mouse was euthanized in accordance with the protocol approved by the University of Washington's Institutional Animal Care and Use Committee. None of our adult mice of either sex experienced CTCs that mandated early euthanasia, but seven aged female mice and one aged male mouse did (Figure 3.1 a and b). Convulsions were only observed at doses of 2.0 mg/kg or greater, regardless of sex (Table 3.1; Figure 3.1 a and b).

We used a survival analysis to examine the association of dose and sex with CTCs in more detail. We excluded age because CTCs were not observed in any adult mice. We modeled dose- and sex-associated CTC hazard ratios (HRs) using cox proportional hazard (CPH) regression. We first investigated a model with dose-sex interaction, but ultimately decided against it because of small sample size and concern for overfitting. We subsequently assessed the model with both dose and sex included as independent predicter variables; this model indicated a significant effect of dose, but not of sex ($p=0.0062$ and $p=0.28$, respectively; Table 3.S1), and the likelihood ratio test (LRT) did not indicate that including sex in the model significantly improved fit beyond that of the dose-only model ($p=0.24$; Table 3.S2). This suggests that dose is the strongest predicter of CTC occurrence in aged mice, and we selected the dose-only model, accordingly.

That model estimates that a 1 mg/kg increase in DA dose is associated with a 16-fold increased risk for CTC occurrence (HR = 16.3 [2.9, 90.4], p=0.0015; Table 3.S1).

Our inability to observe an association of sex with CTC occurrence was likely influenced by the fact that no male mice were exposed to the highest DA dose (2.5 mg/kg bw DA), a dose at which 75% of female mice showed CTCs (Figure 3.1). Overall CTC occurrence in males was therefore quite low.

*3.3.2. Association of Dose, Sex, and Age with Hindlimb Tremor Risk*

At every DA dose, aged mice exhibited hindlimb tremors (HLTs) at comparable or greater rates than their adult counterparts. In addition, female aged mice exhibited HLTs at comparable or greater rates than aged males (Figure 3.1 c and d). The lowest observed adverse effect levels (LOAELs) for aged mice were lower than those for adults of the same sex, and LOAELs reported for adult females were lower than those for adult males (Table 3.1). Aged females and males both had LOAELs of 1 mg/kg bw (Table 3.1).

We used CPH regression methods of survival analysis to examine our HLT data. Unlike for CTCs, because both adult and aged animals exhibited HLTs, we were able to include age, sex, and dose as independent variables. Regression models with three- and two-way interactions failed to show significant interaction effects (Table 3.S3), and, as in the case of CTCs, raised concerns about overfitting due to small sample size. We therefore examined additive models, with dose, age, and sex as independent variables without interaction. Across all models explored,

an independent effect of dose was consistently indicated (Table 3.S4). The fit of the full model including all three variables was significantly improved as compared to all nested models (see Table 3.S4 for LRT p-values comparing models with and without variables in turn). That model including dose, age, and sex as independent variables with no interaction indicated that, adjusting for each of the two remaining variables, a 1 mg/kg increase in DA dose was associated with an almost 4-fold greater risk of HLTs (HR = 3.74 [2.00, 6.99], $p=3.6e^{-5}$), aged animals had a three-fold greater risk of HLTs (HR = 3.07 [1.30, 7.24], p=0.010), and females had an approximately 30% greater risk of HLTs (HR = 0.29 [0.11, 0.81], p=0.019; Table 3.S3). Analysis of Schoenfeld residuals suggests that the sex variable, however, may violate the proportional hazard assumption of CPH models, meaning that, though no interactions between sex and other variables were indicated (Table 3.S3), the HRs reported here should be interpreted as weighted averages of the two sex's true hazard ratios over the observation period.

### *3.3.3. Association of Dose and Age with Mean and Maximum Seizure Scores*

Over the course of 90 minutes following DA injection, we observed mice for 1-minute periods at each of the 1-, 5-, 10-, 30-, 40-, 50-, 60-, and 85-minute marks, scoring their seizure-related symptoms on a scale from 0-5 (see section 3.5.6 for more details). Data from 86 mice were included in a subsequent analysis of the mean and maximum seizure scores recorded for each mouse during the periods.

Mean seizure scores, indicative of both severity and persistence of symptoms experienced, ranged from 0 to about 2 in female mice (Figure 3.2 a). Initial two-way ANOVA with dose, age, and the dose and age interaction as independent variables did not indicate a statistically significant interaction effect ($F(1,23)=1.11$, $p=0.30$). We subsequently ran a two-way ANOVA with just dose and age as independent variables and found a significant main effect of age ($F(1,24)=24.49$, $p=4.7e^{-5}$) but not of dose ($F(3,24)=0.87$, $p=0.47$). The results of post hoc pairwise t-tests indicated significantly higher mean seizure scores for the aged female mice compared to adult female mice dosed at 1.5 mg/kg bw DA ($p=0.00011$; Figure 3.2 a).

Trends were nearly identical for maximum seizure scores in female mice (Figure 3.2 b). Two-way ANOVA with interaction again did not indicate a significant interaction effect ($F(1,23)=0.33$, $p=0.57$). Subsequent two-way ANOVA without interaction again indicated an effect of age on maximum seizure score, but not of dose ($F(1,24)=16.85$, $p=0.00040$ and $F(3,24)=1.89$, $p=0.16$, respectively). Finally, as for mean seizure score, post hoc pairwise t-tests identified significantly greater maximum seizure scores for aged female mice compared to adult female mice administered 1.5 mg/kg bw DA ($p=0.0078$; Figure 3.2 b).

As for females, mean seizure scores for male mice ranged from 0 to about 2 (Figure 3.2 a). Two-way ANOVA with interaction did not identify a significant effect of an age and dose interaction on mean seizure score ($F(3, 31)=0.079$, $p=0.97$). However, main effects of both age and dose were identified in an additive ANOVA ($F(1,34)=6.77$, $p=0.014$ and $F(3,34)=2.90$,

p=0.049, respectively). Pairwise t-tests did not, however, identify significant differences in direct comparisons.

Finally, two-way ANOVA with interaction did not identify a significant effect of an age and dose interaction for maximum seizure scores for male mice ($F(3, 31)=0.554$, $p=0.65$; Figure 3.2 b). ANOVA without interaction showed no evidence for a main effect of age ($F(3,34)=0.994$, $p=0.41$), but there was evidence for a near significant effect of dose ($F(1,34)=3.56$, $p=0.068$).

### *3.3.4. Association of Dose and Age with Concentrations of DA in Tissues*

We quantified DA concentrations (wet weight) in the serum, cortex, and kidneys of all mice at the time of their euthanasia. As expected, tissue DA concentrations in all 20 control animals administered saline were below detection limits. Data from 68 remaining animals that had been exposed to DA were analyzed for trends in tissue DA concentrations.

#### 3.3.4.1. Concentrations of DA in Female Mouse Tissues 90 Minutes Post Exposure

Domoic acid concentrations in the serum of female mice at 90 minutes post-injection ranged from just over 400 ng/g to levels below detection (Figure 3.3). Our initial two-way ANOVA assessing the effect of dose, age, and the dose and age interaction on DA concentrations in the serum did not indicate a statistically significant interaction effect ($F(1,23)=0.728$, $p=0.40$). Our additive ANOVA indicated a near significant effect of age on serum DA concentrations ($F(1,24)=4.04$, $p=0.056$), and no effect of dose ($F(3,24)=0.62$, $p=0.55$).

Serum DA concentration was not associated with mean seizure score in either adult or aged females (Figure 3.S1).

Domoic acid concentrations in the right cortices of female mice euthanized 90 minutes after DA injection were all less than 30 ng/g, and 15 were below detection limits; these are noticeably lower than concentrations in serum (Figure 3.3). Initial two-way ANOVA assessing the effect of dose, age, and the dose and age interaction on DA concentrations in cortices did not indicate a statistically significant interaction effect ($F(1,24)=2.83$, $p=0.11$). Additive ANOVA indicated a significant main effect of age ($F(1,25)=21.26$, $p=0.00011$) but not of dose ($F=(3,25)=0.40$, $p=0.76$). Post hoc pairwise t-tests indicated significantly higher cortical DA concentrations for aged females compared to adult females at the 1.5 mg/kg dose ($p=0.0037$; Figure 3.3).

Kidney tissue from female mice had the highest concentrations of DA (Figure 3.3). As for both serum and cortex, initial two-way ANOVA assessing the effect of dose, age, and the dose and age interaction on DA concentrations in kidney tissue did not indicate a statistically significant interaction effect ($F(1,24)=0.63$, $p=0.43$). As for cortex, age was associated with kidney DA concentrations in the additive ANOVA ($F(1, 25)=10.86$, $p=0.0029$) but dose was not ($F(3, 25)=2.40$, $p=0.092$). Post hoc pairwise t-test showed significantly higher kidney DA concentrations in aged female mice compared to adult female mice at the 1.5 mg/kg dose ($p=0.018$).

3.3.4.2. Concentrations of DA in Male Mouse Tissues 90 Minutes Post Exposure

In general, the concentrations of DA measured in tissues from male mice were lower than those measured in tissues from female mice administered comparable doses (Figure 3.3). Age was also less clearly associated with tissue DA concentrations in male mice. For instance, DA concentrations in male mouse serum showed no significant association with dose, age, or a dose and age interaction (additive dose $F(3,34)=0.47$, $p=0.71$; additive age $F(1,34)=1.91$, $p=0.18$; interaction $F(3,31)=0.96$, $p=0.43$). Additionally, all DA concentrations in male cortical samples were below detection limits. For male kidney DA concentrations, initial two-way ANOVA assessing the effect of dose, age, and the dose and age interaction did not indicate a statistically significant interaction effect $F(3,31)=0.69$, $p=0.57$). For the additive model, neither dose nor age was found to be associated with DA concentrations ($F(3,34)=2.50$, $p=0.076$ and $F(1,34)=0.034$, $p=0.85$ for dose and age, respectively). Unlike females, serum DA concentrations did correlate with mean seizure scores in adult male mice, though not in aged males (Figure 3.S1).

3.3.4.3. Concentrations of DA in the Cortices of Mice Euthanized Early

Domoic acid concentrations were also quantified in the six cortical samples from mice euthanized early due to CTCs that contained enough material for analysis. As expected these values tended to be higher than those observed in tissues collected 90 min after DA exposure from mice that did not have to be euthanized early. Cortical DA concentrations in aged female mice euthanized early for CTCs were 41.5 and 28.0 ng/g in two of the mice administered 2.0

mg/kg bw DA, and 64.9, 36.7, and 37.4 ng/g in the three mice administered 2.5 mg/kg bw DA. The DA concentration in the cortex of the one aged male mouse that was euthanized early for CTCs, which had been administered a DA dose of 2.0 mg/kg bw, was 16.9 ng/g.

## 3.4. Discussion

This is the first laboratory study to directly assess age-associated DA toxicity in both female and male mice. We tested gross toxicity and seizure-related symptoms and found consistent evidence that aged mice are more susceptible to acute DA neurotoxicity, and that female mice are more sensitive than males. We also report evidence that aged animals maintain higher concentrations of DA in serum, cortical, and kidney tissue than their adult counterparts. These findings will help to identify risk factors for DA toxicity that should be considered when protecting communities exposed to DA, moving public health research forward in the face of increased HAB threats [12–14].

### *3.4.1. Dose-Dependent Acute Domoic Acid Neurotoxicity*

Evidence for an association between DA dose and neurotoxic endpoints was most clear when analyses could include data from all mice, given all doses. Survival analysis, the most data-inclusive of our techniques, indicated an association between higher DA doses and greater occurrence of both severe and moderate neurotoxic endpoints (CTCs and HLTs, respectively). Furthermore, while we saw significant and near-significant effects of greater DA dose on higher

mean and maximum seizure scores, respectively, in male mice (for whom all dose groups were analyzed), neither score was seen to be associated with DA dose in females, for whom analysis did not include aged mice in the 2.0 and 2.5 mg/kg bw DA dose groups.

Our data also show surprisingly limited evidence for an association between DA dosing and concentrations of DA persistent in mouse tissues at 90 minutes post-exposure; age proved a far stronger predictor of DA concentrations. In the case of females, this may again be due to the need to limit the range of DA doses included in analysis, and to the high variability of DA concentrations in each tissue type for aged animals. In the case of males, this likely results from the fact that tissue DA concentrations were low across the board.

Additionally, the results reported here indicate that future research assessing low-level, subconvulsive DA exposures in aged mice should consider using doses lower than 1.0 mg/kg bw. Though studies in adult mice might suggest that doses over 1.0 mg/kg bw DA are appropriate subconvulsive exposures, these doses can cause moderate neurotoxic symptoms in mice of more advanced age (40% and 20% HLTs in aged female and male mice, respectively). Additionally, doses of 2.0 mg/kg bw DA and above should be especially avoided, as they elicit potentially lethal CTC outcomes (80% and 20% in aged female and male mice, respectively).

### *3.4.2. Age-Associated Susceptibility to Acute Domoic Acid Toxicity*

Our reports of more severe and persistent neurotoxic symptoms following acute DA exposure in aged animals (as compared to adults) and our observations of higher DA

concentrations in tissues from aged mice (at least in the case of our females) are consistent with clinical reports from the 1987 human DA poisoning event, and with the two laboratory studies that have assessed acute DA symptomology in aged animals since then. When age-associated DA susceptibility was first reported in humans in 1987, it was suggested that impaired renal function might mediate the relationship [7], because younger patients that experienced severe symptoms all had preexisting illnesses that involved impaired kidney performance [7]. Later, in 2002, it was reported that hippocampal slices (CA1 region) from young (3 mo) Sprague Dawley rats had some capacity to attenuate DA toxicity with repeated exposures, while slices from aged rats (26-29 mo) did not [15]. Later, in 2007, Hesp et al. followed up on this work and administered DA intraperitoneally (IP) and intrahippocampally (IH) to young (2-3 mo) and aged (22-27 mo) male Sprague Dawley rats [16]. Aged rats demonstrated increased seizure activity and mortality following the IP injections but not the IH administration, suggesting to the researchers that age-associated susceptibility was likely due to reduced toxin clearance, not increased neuronal sensitivity [16]. Thus, the age-associated susceptibility reported here complements the prevailing hypothesis that slower elimination in older animals contributes to their more severe symptoms following acute DA exposure.

### *3.4.3. Sex-Associated Susceptibility to Acute Domoic Acid Toxicity*

Our results point to possible greater DA susceptibility in female mice than in males and suggest that this may be particularly true when comparing mice of advanced age. These results

are surprising given that, though limited, earlier work assessing sex differences in DA susceptibility has generally suggested that males are more sensitive to the toxin [7,17,18]. While our direct comparisons between sexes were constrained by the fact that we tested females and males asynchronously and administered them slightly different doses, our data and previous studies suggest that the possibility of sex differences changing over the lifespan should be studied further.

In the PEI poisoning incident, men were more likely than women to experience hospitalization and memory loss. At the time it was suggested that this might result from behavioral differences between sexes, as opposed to inherent differences in biological susceptibility; researchers speculated that men perhaps only reported illness or sought clinical care when their symptoms were more severe, as compared to women [7]. Then, in a 1991 study concerning psychoneuroendocrine regulation by the lateral septal area (LSA) of the brain, DA was used to induce lesions in female and male Sprague Dawley rats' LSA regions. In addition to their primary outcomes regarding humoral immune responses, the researchers made the unexpected finding that male rats experienced greater cell loss in the LSA following DA infusion, compared to females [17]. This work prompted follow-up in a 2013 study – female and male Sprague Dawley rats were given low doses of DA (0, 1.0, 1.8 mg/kg bw, IP injection), and their behavior was observed for 3 hours [18]. Although both sexes demonstrated an increase in locomotion, grooming activity, and stereotypic behavior, females demonstrated effects earlier

than males [18]. However, overt toxicity (mortality) was more common in the male rats than the female rats [18].

There are multiple, not mutually exclusive, possible explanations for differences in female and male susceptibility to DA poisoning. First, as we discussed for aging, Baron et al.'s report of earlier symptom onset in females may imply meaningful differences in toxicokinetics and elimination pathways between the sexes [18]. The higher DA concentrations that we observed in tissues from female mice support this conclusion. Future work should consider impairment of DA clearance that develops in each sex over the lifespan, to better explore this possibility.

However, factors other than toxicokinetics may play a role in sex differences in DA toxicity and may underlie, specifically, the development of sex differences with advancing age. Endogenous gonadal hormones have been demonstrated to protect rodent brains from excitotoxic injury. In 1999, it was reported that systemic kainic acid (KA) administration induced hilar dentate neuronal damage in castrated males and ovarectomized females. It was additionally shown that impacts on neuronal integrity in intact females varied depending on the point in the estrous cycle at which KA exposure occurred, and that injection of estradiol, progesterone, and estrogen with KA mitigated associated injury at some points in the estrous cycle [19]. Our observation of severe DA neurotoxicity in aged females may therefore be mediated by changes in sex hormones over the lifespan: our aged mice were 25-28 mo and females were likely experiencing advanced age-associated hormone fluctuations [13]. Conversely, the female rats included in previous work, which indicated greater susceptibility in males, were significantly

younger: 14-18 weeks old. Females in earlier work were young enough that they may have experienced protection from higher levels of sex hormones [18]. Thus, it is possible that previous reports of male susceptibility are accurate at young adult and adult life stages, but that hormonal changes at more advanced ages change sex-associated susceptibility. This idea could be investigated further by including more granular age groups in future studies and covering more of the lifespan.

### *3.4.4. Conclusions and Implications for Human Health*

Consistent with previous work, our study strongly suggests that advanced age is associated with greater neurotoxicity following acute exposure to DA. It also suggests that aging-associated impairment of renal function or elimination processes may be partially responsible for this. Unlike the admittedly limited previous literature, though, our study also indicates that female mice are more sensitive to DA neurotoxicity than males. Because this sex difference was observed specifically in our aged animals, we suggest that it may be related to aged females' reduced protection from sex hormones. Sex differences in DA susceptibility, then, will present differently as hormones fluctuate over the lifespan, and may depend on the age at which sexes are compared.

As ocean conditions continue to change, DA contamination of marine ecosystems is likely to increase as warmer ocean temperatures have been linked to geographically larger and longer lasting *Pseudo-nitzschia* blooms [20]. It is essential that we understand which groups

within our communities will be most at risk from rising threats of increased toxin exposure through seafood, so that we can take appropriate protective measures. This study highlights the need to consider multiple life stages, and specifically advanced life stages, as we design such measures. It also shows that sex differences may be more dynamic than previously presumed. Additional research to inform public health actions should include study designs that compare sexes at multiple points in the lifespan.

## 3.5. Materials and Methods

### *3.5.1. Study Design*

Dose-response studies were conducted with 25 adult and 25 aged female mice administered 0-2.5 mg/kg bw domoic acid (DA) between June 2019 and January 2020, and with 25 adult and 25 aged male mice administered 0-2.0 mg/kg bw DA between May and October 2020. On each experimental day, one to six mice underwent testing illustrated in Figure 3.4. In brief, each mouse was taken into a testing room, acclimated to test housing, injected intraperitoneally with saline or DA, and then returned to their test housing and observed both in real-time and via a video recording for 90 minutes. If clonic-tonic convulsions (CTCs) associated with severe DA toxicity occurred and sustained for at least 30 seconds, animals had to be humanely euthanized prior to 90 minutes. Otherwise, all animals were euthanized and dissected at the end of the 90-minute period. All animal handling and experimental procedures were performed in accordance

with protocols approved by the Institutional Animal Care and Use Committee at the University of Washington.

### *3.5.2. Test Animal Care*

Adult (7-9-month-old) female and male and aged (25-28-month-old) female and male C57Bl/6 NIA mice were obtained from the National Institutes of Aging colony and housed in the controlled environment of our institution's animal research facility. Mice were allowed to acclimate to this research facility for at least one week prior to use in dose-response experiments. Upon arrival at the facility and until they were used, mice were housed in groups as large as five, provided free access to a standard rodent diet (PicoLab® Rodent Diet 20, Lab Diet, USA) and water ad libitum, and were on a 12-hour light/dark cycle. Thirty minutes prior to their injection, animals were separated into individual test housing so that they could acclimate. Test housing was identical to facility housing but lacked food and water access as consumption of either would influence toxin excretion rates.

### *3.5.3. Domoic Acid Dosing*

Due to personnel limitations, we were unable to run both sexes simultaneously. Females received doses of 0, 1.0, 1.5, 2.0, and 2.5 mg/kg bw DA. These doses were selected since they were 1) expected to elicit a spectrum of both subconvulsive and convulsive endpoints, based on previous literature and our lab's pilot work ([21–23] and recently reviewed in [8]), and 2) aligned

with the doses used in a previous study in rats [16]. However, because severe CTCs requiring euthanasia occurred in several of our aged female mice given 2.0 and 2.5 mg/kg bw DA, our sample sizes in those two groups were reduced. Therefore, we chose to shift the range of doses administered to male mice downward to 0.5, 1.0, 1.5, and 2.0 mg/kg bw DA.

Stock solutions of DA targeting 1 mg/mL were prepared by diluting >90% pure powdered DA (Sigma Aldrich) in sterile water, and were then quantified by enzyme-linked immunosorbent assay (ELISA) in parallel triplicate dilution series. Dosing solutions of 0.125, 0.1875, 0.25, 0.3125, and 0.375 mg/mL were prepared by further diluting stock in sterile saline (0.9% sodium chloride; Hospira, Inc.). To allow for more accurate dosing, each mouse was weighed on the day of their injection.

*3.5.4. Real-time Observations and Video Recording*

Mice were video recorded for 90 minutes in their test housing immediately after DA injection. They were also observed in real-time so that sustained CTCs greater than 30 seconds, which mandated early euthanasia to minimize suffering, could be identified. All mice were recorded in side-view (Canon VIXIA HF G10), and some mice were additionally recorded in overhead view (GoPro Hero); there was no effect of camera on neurotoxic analyses. Though periodic video stoppage and battery replacement mandated some periods of interruption, the dual-video system provided a nearly continuous video record. Manual recording of activity and seizure state was performed by researchers during any periods in which cameras failed. These

periods were generally brief and occurred with comparable frequency between dose and age groups and are therefore unlikely to have impacted results.

### *3.5.5. Tissue Collection and Quantification of Domoic Acid*

Mice were euthanized by cervical dislocation either at the end of the 90-minute video recording period or following 30 seconds of sustained CTCs. Blood serum, brains (right and left cortices), and kidneys were collected, flash frozen, and stored in a -80°C freezer prior to DA extraction [24].

Domoic acid was extracted from serum, right cortices, and kidneys in the following manner. Serum was mixed 1:3 volume:volume with 50% MeOH and vortexed for one minute to form homogenate [25]. Cortices were pulverized on liquid nitrogen to form powder, then mixed with 50% MeOH in a 1:3 ratio by weight to form homogenate [25]. Kidneys were ground in a glass mortar and pestle with 50% MeOH in a 1:3 ratio to form a similar homogenate [25]. All homogenates were then spun at 12,000 rpm (13,870 rcf) for 3 min in a desktop centrifuge (Fisher Scientific accuSpin Micro 17) and the supernatant extract was stored in air-tight vials at -4°C until ELISA quantification of DA.

Directly prior to DA quantification, supernatant extracts were removed from -4°C storage and filtered through spin filters (Millipore Ultra-Free MC-GV centrifugal filters) spun at 13,870 rcf (max) for 3 min in a desktop centrifuge (Fisher Scientific accuSpin Micro 17). Then, extracts were diluted 10-fold in dilution buffer [25]; this minimum dilution was chosen to eliminate

matrix effects. Finally, concentrations of DA in nanogram/g (ng/g) were quantified in extracts using commercially available ELISA kits (Biosense® and Abraxis®) as per kit instructions. Detection limits for DA in sample material were 2 ng/g for Biosense® kits and 6.8 ng/g for Abraxis® kits; to ensure consistency, a 6.8 ng/g detection limit was enforced on all quantifications, regardless of kit.

### *3.5.6. Video Recordings Post Exposure*

All videos collected during the post-exposure period were scored by a single researcher blinded to the animal's treatment. Videos were reviewed for seizure-related activity (scored on a 0-5 modified Racine scale; Table 3.2) during 1-minute periods at 1, 5, 10, 30, 40, 50, 60, and 85 minutes post-exposure [16,18,26]. For each 1-minute period, the following outcomes were recorded: 1) presence of subconvulsive hindlimb tremors (HLTs), 2) if present, the number of individual HLTs that occurred, and 3) the maximum seizure score that occurred within the 1-minute period (Table 3.2).

*3.5.7. Statistical Analysis of Behavioral Endpoints*

3.5.7.1. Statistical Analysis of Binary Behavioral Endpoints: Clonic-Tonic Convulsion and Hindlimb Tremor Occurrence

CTC and HLT lowest observed adverse effect levels (LOAELs) were determined as the lowest DA dose after which at least one mouse in each age and sex group experienced a CTC or HLT, respectively.

Because mice were observed for different lengths of time, depending on whether or not they experienced CTCs, we conducted survival analysis on our CTC and HLT data using Cox proportional hazard (CPH) regression [27]. For CTC analysis, observation of a CTC was recorded as an outcome event at the time it occurred, and no observation of a CTC was recorded as a censoring event (end of observation) at 90 minutes. For HLT analysis, observation of an HLT was recorded as an outcome event at the first 1-minute observation period during which an HLT was seen, and no observation of an HLT was recorded as a censoring event at the time observation ended (either the time of euthanasia, if a CTC but no HLT was observed, or 90 minutes).

We generated multivariable and single-variable CPH regression models with independent variables of interest (age, sex, dose, and possible interactions), and compared their fits with the likelihood ratio test. Exponentiated coefficients from the CPH regressions are reported as CTC or HLT hazard ratios (HRs) associated with each variable. We reviewed Schoenfeld residuals and deviance residuals to evaluate proportional hazard assumptions and possible outliers for each

selected CPH model. Because survival analysis struggles to appropriately model situations in which outcomes are rare or nonexistent, we could not estimate the age-associated hazard ratio for CTCs (no CTCs were observed in adult mice, see section 3.3.1). All analyses were performed using the statistical program R (R Core Team 2021).

3.5.7.2. Statistical Analysis of Continuous Behavioral Endpoints: Mean and Maximum Seizure Score

To capture information about the duration and severity of overall seizure-related responses that our mice experienced, we calculated mean and maximum seizure scores for each mouse, across all of their eight 1-minute observation periods (see section 3.5.6). Because females and males were not run concurrently and were administered different ranges of doses (1.0-2.5 mg/kg bw DA for females, and 0.5-2.0 mg/kg bw DA for males), we analyzed results from females and males separately.

Two-way ANOVAs were performed to analyze the effects of dose and age with and without an interaction on mean and maximum seizure scores. We ran Shapiro-Wilk tests and reviewed QQ plots and residuals versus fit plots to evaluate ANOVA assumptions. Where ANOVAs indicated significant interaction effects or main effects of a variable, post hoc pairwise t-tests with Bonferroni correction for multiple comparisons were performed, assessing any differences between the adult and aged mice at each dose. All analyses were performed using the statistical program R (R Core Team 2021).

Because ANOVAs do not accommodate censoring, we could only include animals that had been observed during all eight 1-minute observations periods in these analyses. This necessitated the exclusion of eight mice that were euthanized early and led to the exclusion of female groups administered 2 and 2.5 mg/kg bw DA from the analysis due to small sample sizes ($n<3$). Finally, the video recording of one mouse in the adult female 1 mg/kg bw DA group experienced interruptions, and that mouse was excluded as well.

### *3.5.8. Statistical Analysis of Tissue Domoic Acid Concentrations*

Our statistical analysis of DA concentrations persistent in tissues from adult and aged mice at 90 minutes post-exposure was similar to that for mean and maximum seizure scores, described above. Females and males were analyzed separately. We performed two-way ANOVAs with dose and age as independent variables, with and without interaction. Assumptions were tested with Shapiro-Wilk tests and QQ plot and residuals versus fit plot assessments. Post hoc pairwise t-tests with Bonferroni correction were used to examine age-associated differences within each dose stratum. All analyses were conducted using the statistical program R (R Core Team 2021).

Tissue DA concentration analysis exclusions were the same as those for mean and maximum seizure score data (see section 3.5.7.2), with the exception that the mouse whose video recording was interrupted could still be included in tissue analysis. Grubb's test was used to identify potential outliers in our data from the remaining 69 animals. One outlying serum sample that did not contain enough material for accurate quantification was ultimately excluded from

analysis. Samples below the assay detection limit were assigned a value of 3.4 ng/g DA for the purposes of the analysis; this was half the detection limit of the assay (6.8 ng/g DA, see section 3.5.5).

### 3.6. Funding

This research was funded by the UW Department of Environmental and Occupational Health Sciences, the NOAA Northwest Fisheries Science Center, National Institutes of Health (NIH) R01s ES021930 and ES030319, National Science Foundation (NSF) grants OCE-1314088 and OCE-183904, and the UW NIEHS sponsored Environmental Pathology/Toxicology Training Program (EP/T) Training Grant (NIEHS T32ES007032).

### 3.7. Institutional Review Board Statement

The animal study protocol was approved by the Institutional Animal Care and Use Committee at the University of Washington (protocol 4130-05 approved August 14, 2019-January 31, 2023).

### 3.8. Acknowledgments

This study would not have been possible without the support and involvement of many parties, to whom the authors would like to express their sincere gratitude. First, we would like to thank the staff and animals of the UW Animal Research and Care Facilities and UW Veterinary

Services. Second, huge thanks to Chris Zuidema and Brian High of the UW DEOHS Coders' group for guidance regarding R coding and analysis. Finally, we are grateful to Lars van der Laan, Zhining Sui, Kenneth Rice, and the UW Biostatistics Consulting Service for essential guidance with CPH analysis.

### 3.9. Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of NOAA.

### 3.10. References

1. Zabaglo, K.; Chrapusta, E.; Bober, B.; Kaminski, A.; Adamski, M.; Bialczyk, J. Environmental Roles and Biological Activity of Domoic Acid: A Review. *Algal Res* **2016**, *13*, 94–101, doi:10.1016/J.ALGAL.2015.11.020.
2. Pulido, O.M. Domoic Acid Toxicologic Pathology: A Review. *Mar Drugs* **2008**, *6*, 180–219, doi:10.3390/md20080010.
3. Bates, S.S.; Hubbard, K.A.; Lundholm, N.; Montresor, M.; Leaw, C.P. Pseudo-Nitzschia, Nitzschia, and Domoic Acid: New Research since 2011. *Harmful Algae* **2018**, *79*, 3–43, doi:10.1016/j.hal.2018.06.001.
4. Costa, P.R.; Garrido, S. Domoic Acid Accumulation in the Sardine Sardina Pilchardus and Its Relationship to Pseudo-Nitzschia Diatom Ingestion. *Mar Ecol Prog Ser* **2004**, *284*, 261–268, doi:10.3354/MEPS284261.
5. Costa, P.R.; Rodrigues, S.M.; Botelho, M.J.; Sampayo, M.A. de M. A Potential Vector of Domoic Acid: The Swimming Crab Polybius Henslowii Leach (Decapoda-Brachyura). *Toxicon* **2003**, *42*, 135–141, doi:10.1016/S0041-0101(03)00107-7.

6. Bargu, S.; Powell, C.L.; Coale, S.L.; Busman, M.; Doucette, G.J.; Silver, M.W. Krill: A Potential Vector for Domoic Acid in Marine Food Webs. *Mar Ecol Prog Ser* **2002**, *237*, 209–216, doi:10.3354/MEPS237209.
7. Perl, T.M.; Bédard, L.; Kosatsky, T.; Hockin, J.C.; Todd, E.C.D.; Remis, R.S. An Outbreak of Toxic Encephalopathy Caused by Eating Mussels Contaminated with Domoic Acid. *New England Journal of Medicine* **1990**, *322*, 1775–1780, doi:10.1056/NEJM199006213222504.
8. Petroff, R.; Hendrix, A.; Shum, S.; Grant, K.S.; Lefebvre, K.A.; Burbacher, T.M. Public Health Risks Associated with Chronic, Low-Level Domoic Acid Exposure: A Review of the Evidence. *Pharmacol Ther* **2021**, *227*, doi:10.1016/J.PHARMTHERA.2021.107865.
9. Ferriss, B.E.; Marcinek, D.J.; Ayres, D.; Borchert, J.; Lefebvre, K.A. Acute and Chronic Dietary Exposure to Domoic Acid in Recreational Harvesters: A Survey of Shellfish Consumption Behavior. *Environ Int* **2017**, *101*, 70–79, doi:10.1016/j.envint.2017.01.006.
10. Zheng, G.; Wu, H.; Che, H.; Li, X.; Zhang, Z.; Peng, J.; Guo, M.; Tan, Z. Residue Analysis and Assessment of the Risk of Dietary Exposure to Domoic Acid in Shellfish from the Coastal Areas of China. *Toxins 2022, Vol. 14, Page 862* **2022**, *14*, 862, doi:10.3390/TOXINS14120862.
11. Bargu, S.; Goldstein, T.; Roberts, K.; Li, C.; Gulland, F. Pseudo-Nitzschia Blooms, Domoic Acid, and Related California Sea Lion Strandings in Monterey Bay, California. *Mar Mamm Sci* **2012**, *28*, 237–253, doi:10.1111/j.1748-7692.2011.00480.x.
12. Sellner, K.G.; Doucette, G.J.; Kirkpatrick, G.J. Harmful Algal Blooms: Causes, Impacts and Detection. *J Ind Microbiol Biotechnol* **2003**, *30*, 383–406, doi:10.1007/S10295-003-0074-9.
13. Epstein, P.R.; Chivian, E.; Frith, K. Nature's Services: Societal Dependence on Natural Ecosystems. *Plant Health Prog* **2003**, 10, doi:10.1094/PHP.
14. Young, N.; Sharpe, R.A.; Barciela, R.; Nichols, G.; Davidson, K.; Berdalet, E.; Fleming, L.E. Marine Harmful Algal Blooms and Human Health: A Systematic Scoping Review. *Harmful Algae* **2020**, *98*, 101901, doi:10.1016/j.hal.2020.101901.
15. Kerr, D.S.; Razak, A.; Crawford, N. Age-Related Changes in Tolerance to the Marine Algal Excitotoxin Domoic Acid. *Neuropharmacology* **2002**, *43*, 357–366, doi:10.1016/S0028-3908(02)00088-6.
16. Hesp, B.R.; Clarkson, A.N.; Sawant, P.M.; Kerr, D.S. Domoic Acid Preconditioning and Seizure Induction in Young and Aged Rats. *Epilepsy Res* **2007**, *76*, 103–112, doi:10.1016/j.eplepsyres.2007.07.003.
17. Wetmore, L.; Nance, D.M. Differential and Sex-Specific Effects of Kainic Acid and Domoic Acid Lesions in the Lateral Septal Area of Rats on Immune Function and Body Weight Regulation. *Exp Neurol* **1991**, *113*, 226–236, doi:10.1016/0014-4886(91)90179-G.
18. Baron, A.W.; Rushton, S.P.; Rens, N.; Morris, C.M.; Blain, P.G.; Judge, S.J. Sex Differences in Effects of Low Level Domoic Acid Exposure. *Neurotoxicology* **2013**, *34*, 1–8, doi:10.1016/J.NEURO.2012.10.010.

19. Azcoitia, I.; Fernandez-Galaz, C.; Sierra, A.; Garcia-Segura, L.M. Gonadal Hormones Affect Neuronal Vulnerability to Excitotoxin-Induced Degeneration. *Journal of Neurocytology 1999 28:9* **1999**, *28*, 699–710, doi:10.1023/A:1007025219044.
20. McCabe, R.M.; Hickey, B.M.; Kudela, R.M.; Lefebvre, K.A.; Adams, N.G.; Bill, B.D.; Gulland, F.; Thomson, R.E.; Cochlan, W.P.; Trainer, V.L. An Unprecedented Coastwide Toxic Algal Bloom Linked to Anomalous Ocean Conditions. *Geophys Res Lett* **2016**, *43*, 10,366-10,376, doi:10.1002/2016GL070023.
21. Tryphonas, L.; Truelove, J.; Nera, E.; Iverson, F. Acute Neurotoxicity of Domoic Acid in the Rat. *Toxicol Pathol* **1990**, *18*, 1–9, doi:10.1177/019262339001800101.
22. Truelove, J.; Iverson, F. Serum Domoic Acid Clearance and Clinical Observations in the Cynomolgus Monkey and Sprague-Dawley Rat Following a Single IV Dose. *Bulletin of Environmental Contamination and Toxicology 1994 52:4* **1994**, *52*, 479–486, doi:10.1007/BF00194132.
23. Vieira, A.C.; Martinez, J.M.C.; Pose, R.B.; Queijo, A.A.; Posadas, N.A.; Lopez, L.M.B. Dose-Response and Histopathological Study, With Special Attention to the Hypophysis, of the Differential Effects of Domoic Acid on Rats and Mice. *Microsc. Res. Tech* **2015**, *78*, 396–403, doi:10.1002/jemt.22486.
24. Suzuki, C.A.M.; Hierlihy, S.L. Renal Clearance of Domoic Acid in the Rat. *Food and Chemical Toxicology* **1993**, *31*, 701–706, doi:10.1016/0278-6915(93)90140-T.
25. Frame, E.; Lefebvre, K.A. *ELISA Methods for Domoic Acid Quantification in Multiple Marine Mammal Species and Sample Matrices (NMFS-NWFSC-122)*; 2013;
26. Tasker, R.A.R.; Connell, B.J.; Strain, S.M. Pharmacology of Systemically Administered Domoic Acid in Mice. *Can J Physiol Pharmacol* **1991**, *69*, 378–382, doi:10.1139/y91-057.
27. Mathew, A.; Pandey, M.; Murthy, N.S. Survival Analysis: Caveats and Pitfalls. *Eur J Surg Oncol* **1999**, *25*, 321–329, doi:10.1053/EJSO.1998.0650.

### 3.11. Tables and Figures

**Table 3.1.** Lowest observed adverse effect levels (LOAELs) associated with domoic acid (DA)-induced clonic-tonic convulsions (CTCs) and hindlimb tremors (HLTs) in each age and sex group.

| Group | CTC LOAEL | HLT LOAEL |
|---|---|---|
| Adult females | n/a | 1.5 mg/kg DA |
| Aged females | 2.0 mg/kg DA | 1.0 mg/kg DA |
| Adult males | n/a | 2.0 mg/kg DA |
| Aged males | 2.0 mg/kg DA | 1.0 mg/kg DA |

**Table 3.2.** Modified Racine scale used to score symptom severity during 1-minute observation periods at 1, 5, 10, 30, 40, 50, 60, and 85 minutes post-DA exposure [16,23,26].

| Score | State or Symptoms |
|---|---|
| 0 | No apparent effect |
| 1 | Pressed flat, little movement or stumbling walk |
| 2 | Hunched, head bobbing |
| 3 | Hindlimb tremors |
| 4 | Forelimb tremors and/or wet dog shakes |
| 5 | Clonic-tonic convulsions, rearing and falling, full-body shaking |

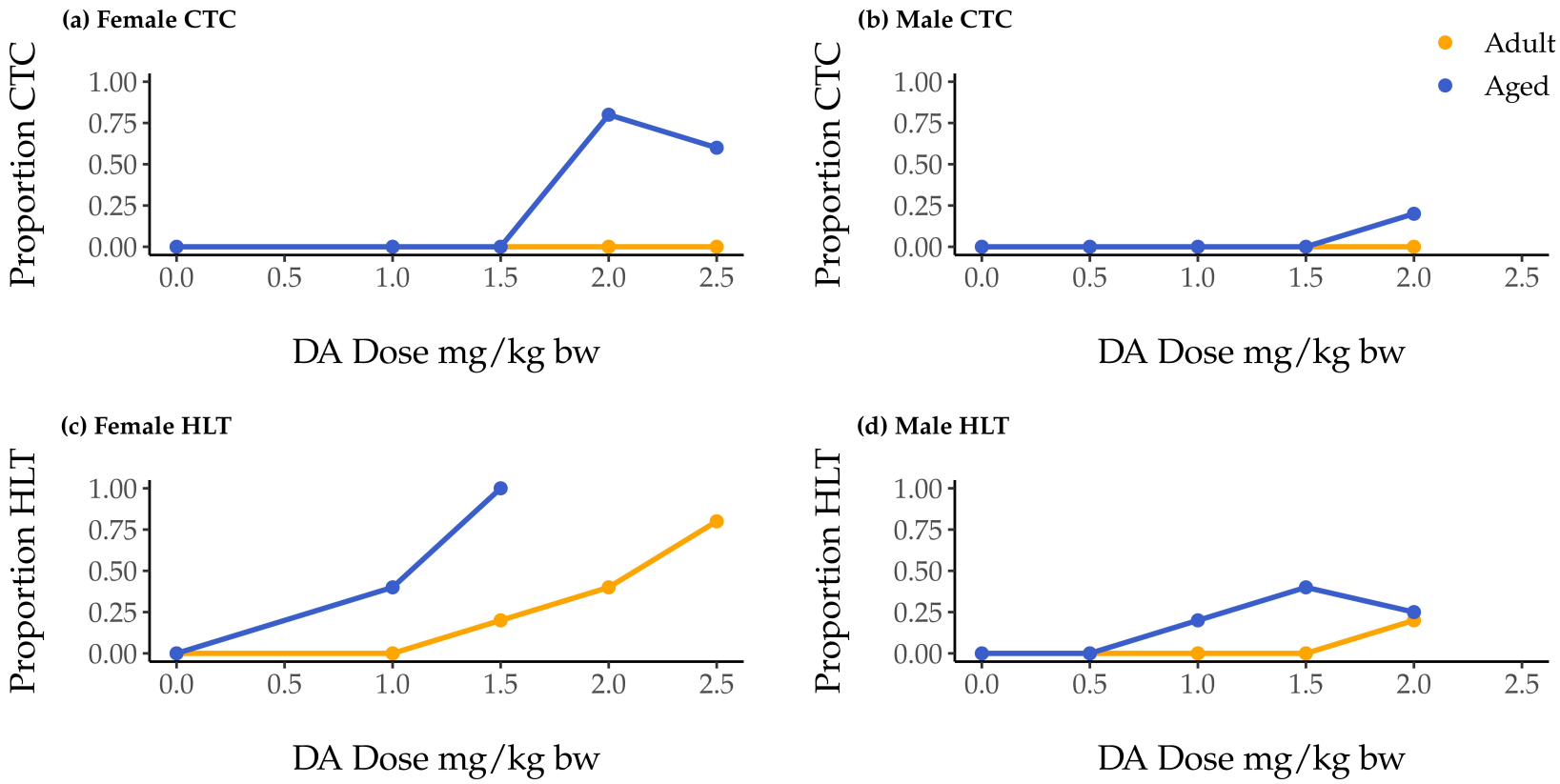


**Figure 3.1.** Proportion of aged (blue) and adult (yellow) female and male mice showing clonic-tonic convulsions (CTCs; a and b, respectively) and hindlimb tremors (HLTs; c and d, respectively) during the 90-minute observation period following intraperitoneal (IP) injection with saline or domoic acid (DA; 1.0-2.5 mg/kg bw for females, 0.5-2.0 mg/kg bw for males). CTC data include all mice; HLT data are presented only for groups that maintained n=3 after the exclusion of mice that could not be observed for a full 90 minutes (i.e., data are not presented for aged females given 2.0 and 2.5 mg/kg bw).

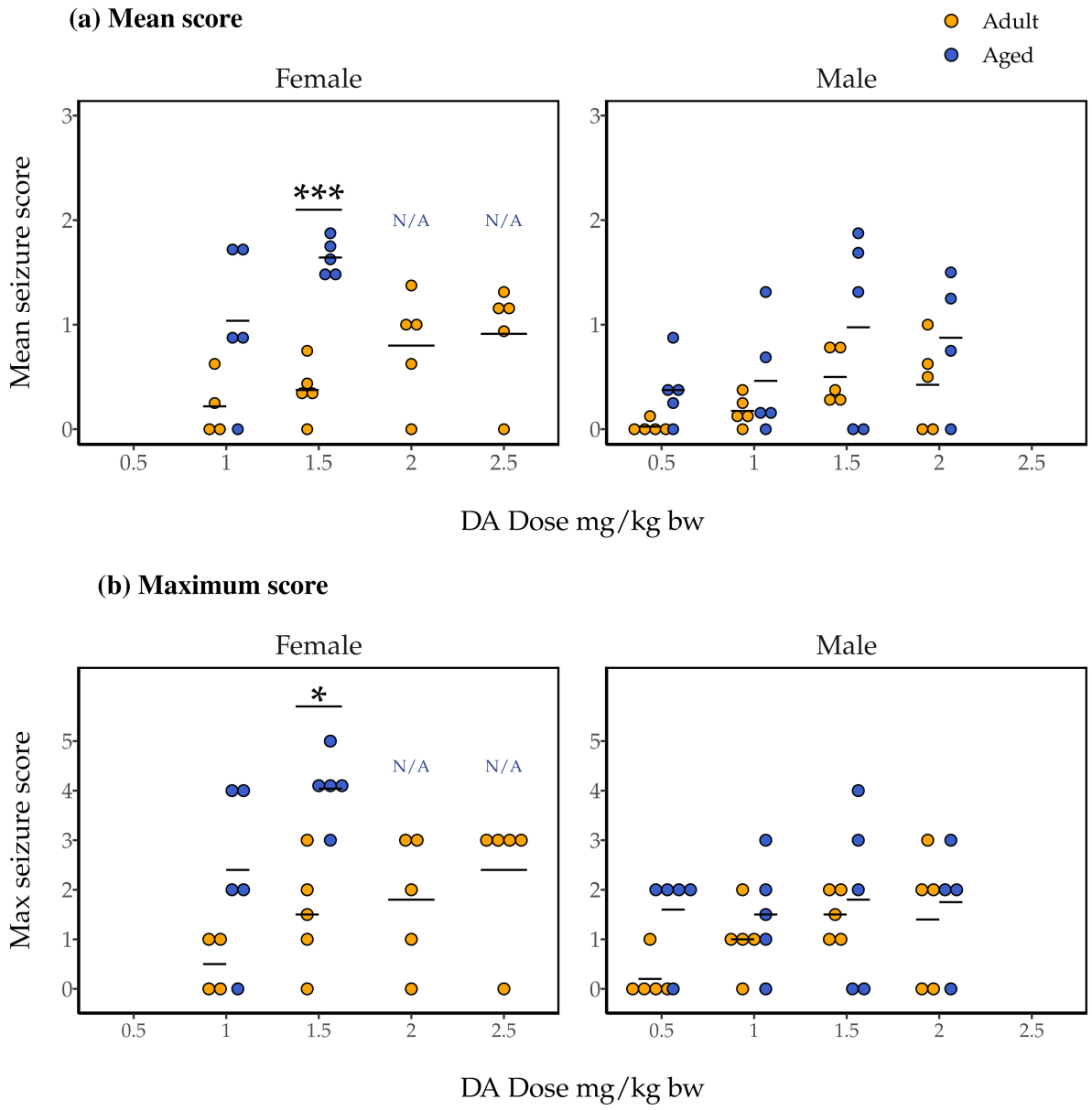


**Figure 3.2.** Mean (a) and maximum (b) seizure scores observed in aged (blue) and adult (yellow) female and male mice during 90 minutes of observation following IP injection with DA (1.0-2.5 mg/kg bw for females, 0.5-2.0 mg/kg bw for males). Data are presented only for groups that maintained n=3 after the exclusion of mice that could not be observed for a full 90 minutes (i.e., data are not presented for aged females given 2.0 and 2.5 mg/kg bw). Results of post-hoc pairwise t-tests comparing adult and aged mice's seizure scores within each sex- and dose-group are shown; *p<0.05, ***p<0.0005.

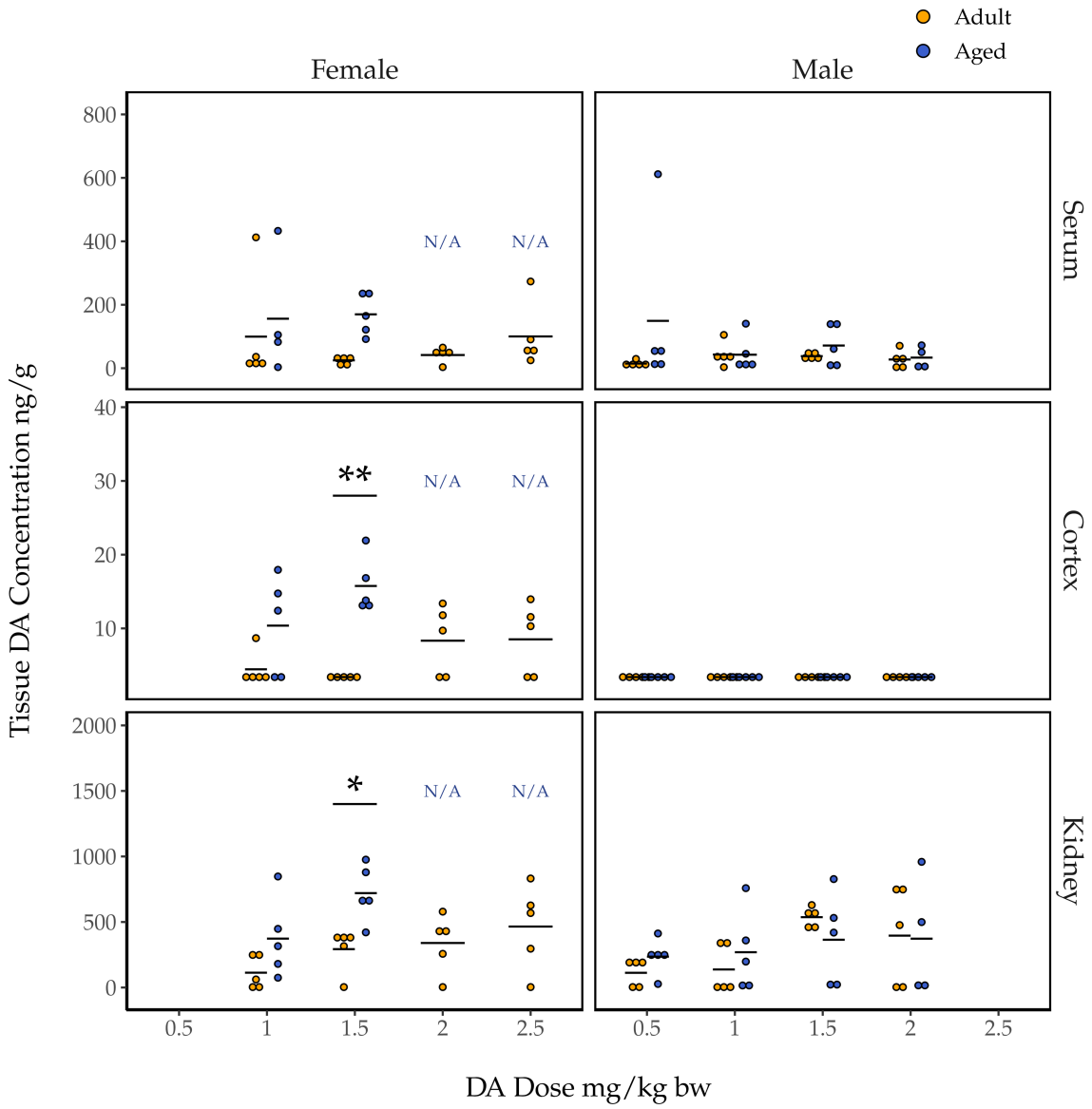


**Figure 3.3.** Domoic acid concentrations (n=4-5; wet weight) quantified in the serum, right cortex, and kidneys of aged (blue) and adult (yellow) female and male mice 90 minutes after IP injection of DA (1.0-2.5 mg/kg bw for females, 0.5-2.0 mg/kg bw for males). Data are presented only for groups that maintained n=3 after the exclusion of mice that were euthanized prior to 90 minutes post-exposure due to sustained CTCs (i.e., data are not presented for aged females given 2.0 and 2.5 mg/kg bw). One serum sample outlier from an aged female mouse in the 1.0 mg/kg group is omitted. Results of post-hoc pairwise t-tests comparing adult and aged mice's tissue DA concentrations within each sex- and dose-group are shown; *$p<0.05$, **$p<0.005$.

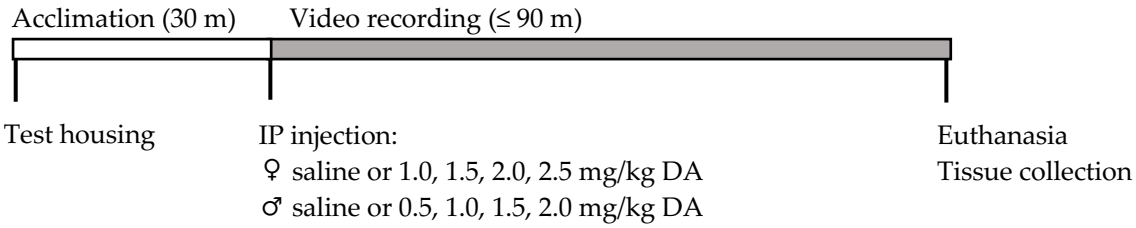


**Figure 3.4.** Day-of-experiment timeline of dosing and testing procedures for each mouse. Mice were brought into the experimental space, separated into and acclimated to test housing, administered saline or DA via intraperitoneal (IP) injection, returned to their test housing, and observed both in real-time and via video recordings for up to 90 minutes. If mice exhibited 30 seconds of sustained clonic-tonic convulsions (CTCs) then they had to be humanely euthanized prior to 90 minutes. Otherwise, mice were euthanized at the end of 90 minutes. All animals were dissected, and serum, brain, and kidney samples were collected.

### 3.12. Supplementary Materials

**Table 3.S1.** Estimated hazard ratios (HRs) and p-values associated with variables included in each Cox proportional hazard (CPH) regression model for clonic-tonic convulsions. *p<0.05.

| Models and model variables | Concordance | Estimated HRs | p-values |
|---|---|---|---|
| Additive | 91% | | |
| Dose | | 10.0 [1.9, 52] | 0.0062* |
| Sex | | 0.29 [0.31, 2.75] | 0.28 |
| Single-variable dose | 90% | | |
| Dose | | 16.3 [2.9, 90.4] | 0.0015 |
| Single-variable sex | 72% | | |
| Sex | | 0.12 [0.015, 1.0] | 0.050 |

**Table 3.S2.** Likelihood ratio test p-values comparing nested single-variable CPH model fit to additive multivariable CPH model fit for clonic-tonic convulsions. ***p<0.0005.

| **Full model** | **Nested model** | **LRT comparing full model to nested model** |
|---|---|---|
| Dose + Sex | Dose | 0.24 |
| Dose + Sex | Sex | 0.00020*** |

**Table 3.S3.** Estimated hazard ratios (HRs) and p-values associated with variables included in each Cox proportional hazard (CPH) regression model for hindlimb tremors. *p<0.05, **p<0.005, ***p<0.0005.

| Models and model variables | Concordance | Estimated HRs | p-values |
|---|---|---|---|
| Interaction | | | |
| Dose * age + sex | 84.5% | | |
| Dose | | 10.0 [2.0, 50.0] | 0.0053* |
| Age | | 36.0 [0.84, 1540.1] | 0.616 |
| Sex | | 0.31 [0.11, 0.87] | 0.026* |
| Dose:age | | 0.29 [0.051, 1.65] | 0.16 |
| Dose * sex + age | 84.9% | | |
| Dose | | 3.6 [1.81, 7.0] | 0.00024*** |
| Age | | 3.1 [1.3, 7.2] | 0.011* |
| Sex | | 0.18 [0.0081, 3.81] | 0.27 |
| Dose:sex | | 1.37 [0.23, 8.065] | 0.73 |
| Sex * age + dose | 85.1% | | |
| Dose | | 3.72 [2.00, 7.00] | $3.9e^{-5}$*** |
| Age | | 2.83 [1.10, 7.25] | 0.030* |
| Sex | | 0.21 [0.025, 1.71] | 0.14 |
| Sex:age | | 1.61 [0.15, 17.44] | 0.70 |
| Additive | | | |
| Dose + age + sex | 85.3% | | |
| Dose | | 3.74 [2.00, 6.99] | $3.6e^{-5}$*** |
| Age | | 3.07 [1.30, 7.24] | 0.010* |
| Sex | | 0.29 [0.11, 0.81] | 0.019* |
| Dose + age | 83.8% | | |
| Dose | | 1.57 [2.47, 9.26] | $3.4e^{-6}$*** |
| Age | | 2.85 [1.21, 6.68] | 0.016* |
| Dose + sex | 82.1% | | |
| Dose | | 1.23 [1.83, 6.45] | 0.00012*** |
| Sex | | 0.32 [0.11, 0.88] | 0.028* |
| Sex + age | 72.0% | | |
| Sex | | 0.20 [0.75, 0.54] | 0.0015** |
| Age | | 2.41 [1.03, 5.64] | 0.043* |

**Table 3.S3.** *continued*

| Single-variable | | | |
|---|---|---|---|
| Dose | 79.1% | | |
| Dose | | 4.43 [2.30, 8.53] | $8.6e^{-6}$*** |
| Age | 60.1% | | |
| Age | | 2.29 [0.98, 5.36] | 0.055 |
| Sex | 67.1% | | |
| Sex | | 0.21 [0.078, 0.56] | 0.0018** |

**Table 3.S4.** Likelihood ratio test p-values comparing nested single-variable CPH model fit to additive multivariable CPH model fit for hindlimb tremors. *p<0.05, **p<0.005, ***p<0.0005.

| **Full model** | **Nested model** | **LRT comparing full model to nested model** |
|---|---|---|
| Dose + Age | Dose | 0.013* |
| Dose + Sex | Dose | 0.018* |
| Age + Dose | Age | $1.3e^{-7}$*** |
| Age + Sex | Age | 0.00036*** |
| Sex + Dose | Sex | $1.4e^{-5}$*** |
| Sex + Age | Sex | 0.036* |
| Dose + Age + Sex | Dose + Sex | 0.0078** |
| Dose + Age + Sex | Dose + Age | 0.011* |
| Dose + Age + Sex | Age + Sex | $3.4e^{-6}$*** |

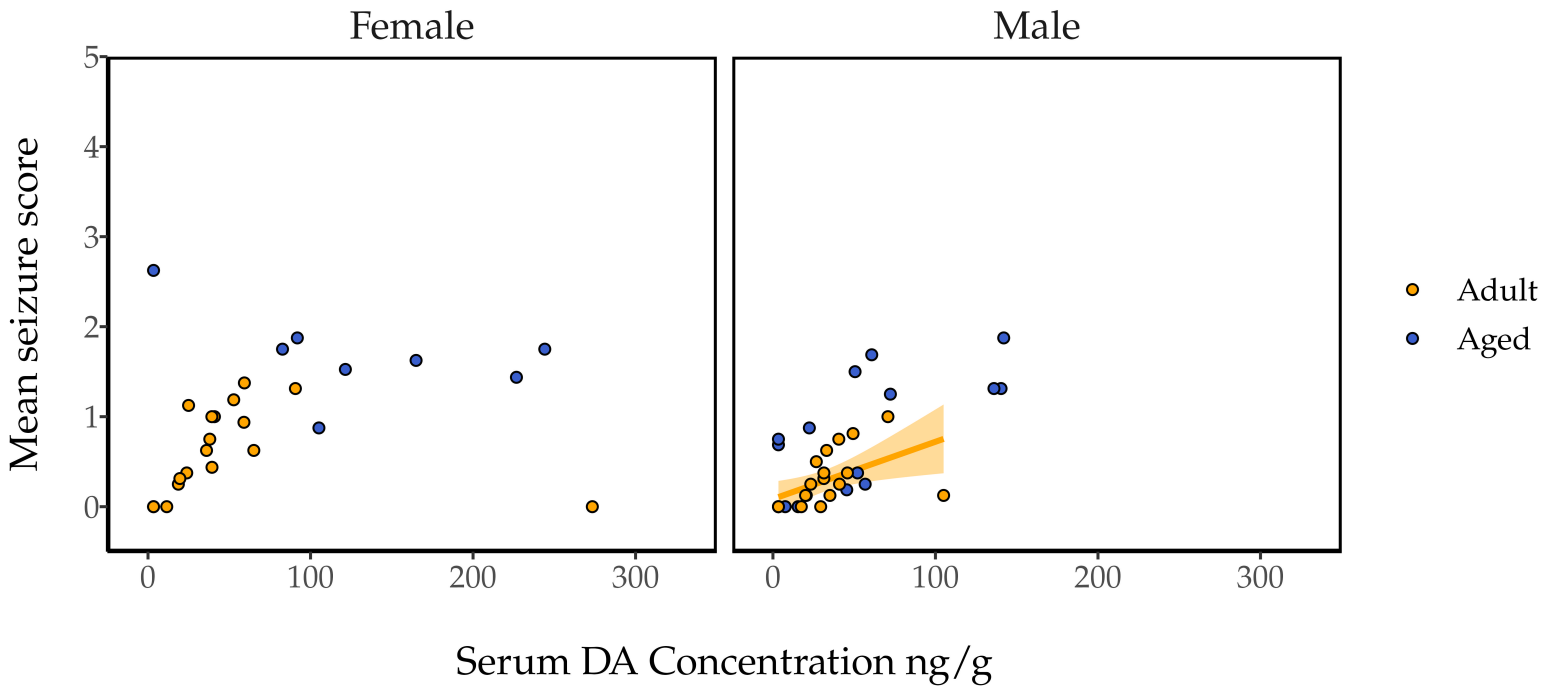


**Figure 3.S1.** Regression analyses of mean seizure scores versus serum DA concentrations indicated that serum DA level was positively associated with mean seizure score in adult male mice only. Overall linear regression model for adult male mice was statistically significant ($F(1,18)=6.51$, $p=0.020$). For every 100 ng/g greater serum DA concentration, mean seizure score increased by 0.64 (± 0.25; $p=0.020$).

# Chapter 4: Behavioral and cardiac impacts of chronic, low-level domoic acid exposure in female mice

## 4.1. Abstract

As harmful algal blooms (HABs) grow in persistence and severity with shifting ocean conditions related to climate change, HAB toxins including the excitatory neurotoxin domoic acid (DA) are becoming more ubiquitous. Concerns mount that consumers might be repeatedly exposed to low levels of DA, and we need to clarify the effects of such exposure on both conventionally DA-associated memory endpoints and peripheral organ systems. This study's original design was to first administer 0.5 mg/kg bw DA to adult and aged female mice thrice weekly for 14 weeks, then assess cardiac function (by echocardiography) and activity levels (in Phenotyper cages) in all animals, then allow a 10-week washout period, and finally repeat the assessments. However, aged mice given DA experienced unexpectedly high rates of mortality and were not able to be included in cardiac and activity tests. We therefore performed echocardiography on all adult mice and on aged control mice in week 13 and then sacrificed our aged animals. Adult mice completed an additional, final week of exposure, then activity assessments, the planned washout period, and final rounds of tests. Mortality was significantly associated with DA exposure in our aged animals – only 30% of our aged mice administered DA survived exposure. We also report subtle but persistent effects of DA exposure on cardiac function and activity: adult mice given DA showed diastolic dysfunction at 13 weeks that phenocopied natural aging impairment, as well as heightened levels of activity. These DA effects remained post washout. These findings show that low levels of DA can have behavioral and cardiac impacts that are persistent after exposure has ended and highlight the need to include activity and cardiac endpoints in research moving forward.

**Keywords:** domoic acid; chronic exposure; aging; cardiac dysfunction

## 4.2. Introduction

The excitatory neurotoxin domoic acid (DA) is produced by diatoms in the genus *Pseudo-nitzschia* during harmful algal bloom (HAB) events, when algal biomass and toxins are rapidly produced in large quantities. Once filter feeding organisms consume DA, it can contaminate a whole food web through trophic transfer [1–5], causing an acute toxicosis in higher-level consumers known as Amnesic Shellfish Poisoning that is characterized by gastrointestinal distress, cognitive impairment, confusion, and, in severe cases, death [6,7]. As the global population whose diet depends on marine systems nears three billion [8], we must develop our understanding of this seafood-borne toxin and its potential threats to public health.

Many recent studies suggest DA will be more consistent and persistent in food webs across a broader geographic range in years to come [9–14], due to increases in HAB occurrence and severity as ocean conditions shift with climate change [15–17]. They also suggest that, unlike the acute exposures that have been the focus of most DA research, chronic exposures to low levels of DA may be increasingly common [18]. Repeated consumption of DA at levels below those that cause overt epileptic symptoms has already been shown to impact human health: reports from the Communities Advancing the Studies of Tribal Nations Across the Lifespan (CoASTAL) cohort describe associations between regular consumption of razor clams, a vector for DA, and poorer performance on certain memory tests [19–21]. While some laboratory rodent and nonhuman primate models have followed up on this work – reporting changes in activity levels, memory function, motor coordination, and aggression depending on exposure regimen [22–25] – literature in the area is still quite limited. Interestingly, some subtle

effects of repeated DA exposure may be reversible; the memory impairments (assessed by radial water tread maze) and activity changes (assessed by open field locomotion) in mice exposed to subconvulsive DA for 25 weeks in a 2017 study were mitigated by a 9-week washout period [26].

In addition to assessing the relationship between chronic DA exposure and neurological function, assessments of possible peripheral organ toxicities are necessary. Organs as diverse as the heart, kidney, liver, lung, reproductive organs, and spleen express the glutamate receptors that DA binds to and activates, and could therefore plausibly be impacted by exposure to DA [30–32]. Finally, susceptibility factors like advanced age that contribute to greater injury following acute DA exposure [6,27] should be studied, since we know the elderly are well represented in groups at risk for regular DA exposure [28,29].

This study explores novel chronic, low-level DA exposure scenarios, and introduces the possibility of peripheral organ system toxicities due to these exposures. Both behavioral (activity level) and cardiac (diastolic and systolic function) endpoints were assessed in adult and aged female C57Bl/6 J mice exposed to chronic, low levels of DA. Findings address possible dual pathologies in novel scenarios of chronic exposure to DA.

## 4.3. Methods

### *4.3.1. Study Design*

Between April and October of 2021, adult and aged female mice were administered 0.5 mg/kg bw DA 3x/week for a total of 14 weeks (Figure 4.1). After 13 weeks of exposure adult and aged mice underwent echocardiography to measure cardiac function. Because mortality in aged mice was higher than expected during both the exposure period and the echocardiography

procedure, aged mice were euthanized by cervical dislocation at this point, and their tissues were collected and either flash frozen or preserved. Adult mice, all of whom survived the first echocardiography, completed a 14$^{th}$ and final week of saline or DA exposure and were monitored for 66 hours in Phenotyper activity cages during week 14. They were then allowed a depuration period of 10 weeks, during which no saline or DA was administered. At the end of that period, Phenotyper monitoring and echocardiography were performed again. At the conclusion of these tests, adult mice were weighed, euthanized by cervical dislocation, and tissues were collected.

*4.3.2. Test Animal Care*

Adult (13 mo at the start of experiments) and aged (24 mo at the start of experiments) female C57/Bl6 J mice were obtained from the Jackson Laboratory and housed in the controlled environment of the University of Washington animal research facility in rooms kept at 21°C. Mice were allowed to acclimate to this research facility for at least one week prior to experimental testing. During acclimation and throughout the experiment, animals were provided free access to a standard rodent diet (PicoLab® Rodent Diet 20, Lab Diet, USA) and water *ad libitum*, and were on a 12/12 hour light/dark cycle. Animals were observed at least twice per week, and mice with emergent, serious health conditions (e.g., tumors impairing respiration, moribundity) were removed from the study and euthanized to minimize suffering. All animal handling and experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Washington.

*4.3.3. Domoic Acid Dosing*

Mice were administered ~0.5 mg/kg bw intraperitoneally (IP) DA 3x/week, with 1-2 days in between each dose. Injections were performed by trained researchers in the lab and by trained technicians with the UW In Vitro Services Core. Stock solutions of DA targeting 1 mg/mL were prepared by diluting >90% pure powdered DA (Sigma Aldrich) in sterile water and quantified by enzyme-linked immunosorbent assay (ELISA) in parallel triplicate dilution series. Dosing solution of 0.0875 mg/mL DA was then prepared by further diluting stock in sterile saline (0.9% sodium chloride; Hospira, Inc.). Syringes were prepared with a correct volume of dosing solution to inject each mouse with a dose between 0.43 and 0.53 mg/mL DA, without injecting any mouse with a volume greater than 0.4 mL. To maintain appropriate dosing throughout the duration of the exposure period, mice were weighed and their dose volumes were calculated weekly. Syringes prepared with dosing solution were stored at 4°C until the day of their use, at which time they were transferred in a dark, sealed container and stored at room temperature for 0-6 hours prior to injection.

*4.3.4. Echocardiography*

In the 13$^{th}$ week of the exposure period, and again after the washout period for adult mice, mice were anaesthetized and underwent echocardiography as described in Chiao et al., 2020. Briefly, mice were anaesthetized by 0.5-1% isoflurane and an echocardiogram was performed using a Siemens Acuson CV-70 equipped with a 13 MHz probe. The ratio of early to late diastolic mitral annulus velocities (Ea/Aa), a measure of diastolic function, was calculated for each mouse. Fractional shortening (FS), a measure of systolic function, was also calculated

for each mouse. All echocardiography, compilation of echocardiography data, and analysis of these data was performed by Sophia Liu of the UW Translational Bioenergetics Lab.

*4.3.5. Phenotyper Activity Cage Testing*

After echocardiography, adult mice were given saline or DA for a 14$^{th}$, final week. In that week and again after the 10-week depuration period, mice were placed in individual Phenotyper activity cages (Noldus 16-cage PhenoLab system) and video-recorded for 66 hours. During this time the mice had free access to a standard rodent diet (PicoLab® Rodent Diet 20, Lab Diet, USA) and water *ad libitum*, and were on a 12/12 hour light/dark cycle. Ethovision XT16 software then analyzed the cages' 66-hour videos, detecting individual mouse location and movement (three-point detection of tail, nose, and center point). The software reported the distance each mouse traveled, the time they spent in "high mobility," "mobility," and "immobility" states (determined by rate-of-change of velocity), time spent in open field and thigmotaxis, and time spent in proximity to (i.e., within 3 cm of) food, water, and shelter.

*4.3.6. Statistical Analysis of Mortality During the Experiment*

Mortality rates in adult and aged mice exposed to DA or unexposed controls were compared by Fisher's exact test, looking for a) an overall association of age and mortality, and b) an overall association of DA exposure and mortality. We then used the Breslow-Day test to assess whether mortality's association with age was different in saline- and DA-exposed mice, and whether DA exposure's association with mortality was different in adult and aged mice. Finally, where the Breslow-Day test indicated significant differences in associations, we used

individual post hoc Fisher's exact tests to examine these group-wise associations directly. All analyses were performed using the statistical program R (R Core Team 2021).

### *4.3.7. Statistical Analysis of Echocardiography*

Data from the echocardiography tests performed in week 13 were analyzed via multiple t-tests. Ea/Aa ratios indicative of diastolic function were compared a) between saline- and DA-exposed adult mice in the 13$^{th}$ week, to assess the effect of DA exposure on cardiac function in adult mice; b) between saline- and DA-exposed adult mice after washout, to assess the impact of that washout period on the cardiac dysfunction observed at 13 weeks (in the first t-test); and c) between saline-exposed adult and aged mice in the 13$^{th}$ week, to assess the impact of age on cardiac function, and ultimately to indicate the extent to which effects of DA exposure may phenocopy the natural effects of aging. Comparisons "a" and "c" above were repeated for FS, indicative of systolic function. Additionally, t-tests were used to compare the body weights and heart weights of DA- and saline-exposed adult mice at the end of the experiment. These analyses were performed by Sophia Liu and the UW Translational Bioenergetics Lab using the statistical program PRISM 8.

### *4.3.8. Statistical Analysis of Phenotyper Cage Activity*

Three-way repeated measures analysis of variance (RM ANOVA) was used to compare the effect of DA exposure and test round (post exposure versus post washout) on activity and anxiety-related endpoints in adult mice, with time of day included as an additional independent variable likely to impact activity levels in these nocturnal animals. Because visual inspection of the data did not show large variation in activity metrics across successive hours within each day

and night, we binned measurements of each endpoint from each of the 66 hours monitored into overall 'night' and 'day' categories for each mouse for ease of analysis. Where three-way RM ANOVA indicated three- or two-way interactions, we followed up with post hoc two- and one-way RM ANOVAs at each distinct level of the interaction terms, respectively. Main effects are reported in the cases where no interaction terms are indicated. All behavioral analyses were performed using the statistical program R (R Core Team 2021).

## 4.4. Results

### *4.4.1. Mortality During the Experiment*

Mortality rates in DA-exposed mice were noticeably greater than mortality rates in unexposed mice (40% compared to 17%, Table 4.1). Though there was not an overall effect of DA exposure on mortality (Fisher's exact test, $p=0.095$), a Breslow-Day test indicated that the DA effect differed between age groups ($p=0.032$), and follow-up Fisher's exact tests showed that this was because DA exposure was not clearly related to mortality in adult mice ($p>0.9$) but was associated with mortality in aged mice ($p=0.038$).

Across DA exposures, rates of attrition in adults were significantly lower than in aged animals (Fisher's exact test, $p=0.00076$, Table 4.1). This was driven by an age-mortality association in the DA-exposed animals (Fisher's exact test, $p=0.00092$), not in the saline-exposed ones (Fisher's exact test, $p>0.9$, Breslow-Day test $p=0.054$).

Of the nine aged animals that did not survive 13 weeks of DA exposure, euthanasia of two was definitively associated with DA exposure (i.e., following CTCs directly after DA injection, both during the fourth week of DA exposure). Mortality in all other mice either was

prompted by the emergence of underlying disease that necessitated euthanasia, or occurred outside of observation periods, for undetermined reasons.

*4.4.2. Association of DA Exposure and Age with Cardiac Function*

Twelve of the adult mice administered saline and twelve of the adult mice administered DA survived both the exposure and the depuration period. These mice underwent echocardiography at both time points. Diastolic function in adult mice given DA was impaired at the end of the exposure period, compared to function in adult mice given saline (decreased Ea/Aa ratio, t-test $p<0.05$; Figure 4.2 a). Further, the observed diastolic dysfunction did not improve with washout; Ea/Aa ratios remained unchanged after the washout period weeks (Figure 4.2 b).

Aged mice administered DA did not survive the first 13 weeks of DA exposure period in high enough numbers to allow for comparison with their saline counterparts. Aged saline mice still underwent echocardiography at 13 weeks. These unexposed aged mice showed diastolic dysfunction that was comparable to the dysfunction observed in adult mice exposed to DA for 13 weeks (Figure 4.2 c).

Neither DA exposure nor age were observed to impact systolic function (Figure 4.3). Additionally, there were not significant differences between body weights or heart weights of DA-exposed and saline-exposed adult mice at the end of the experiment (t-tests, $p>0.05$ for each).

*4.4.3. Association of DA Exposure and Phenotyper Cage Behavioral Endpoints*

Three-way RM ANOVA indicated significantly greater distances traveled by mice at night ($F(1,22)=201.2$, $p=1.5e^{-12}$), and those administered DA ($F(1,22)=4.4$, $p=0.048$). ANOVA

did not indicate a main effect of testing round (i.e., end of exposure versus end of washout) on distance traveled ($F(1,22)=0.22$, $p=0.64$), nor any three- or two-way interactions between the independent variables (Table 4.2).

We ran separate three-way RM ANOVAs to see the effect of our three independent variables on time spent in each of the three mobility states (Table 4.3). For time in the high mobility state, ANOVA analysis showed a significant two-way interaction between treatment and time of day; follow-up one-way ANOVAs indicated that DA treatment was associated with more time in the high mobility state during both day ($F(1,22)=9.1$, $p=0.0064$) and night ($F(1,22)=11.0$, $p=0.0031$). Analysis of time in the middle mobility state did not show evidence of three- or two-way interactions, but indicated main effects of treatment – mice exposed to DA spent more time "mobile" than controls – and time of day – mice were "mobile" for longer at night ($F(1,22)=5.4$, $p=0.030$ and $F(1,22)=68.9$, $p=3.2e^{-8}$, respectively). The only significant effect for immobility was a main effect indicating mice were determined "immobile" for longer periods at night as well ($F(1,22)=155.7$, $p=1.9^{-11}$).

Our analysis of the effects of treatment, time of day, and testing after exposure versus after washout on time spent in each region of the cage environment showed a few associations (Table 4.4). Analysis indicated that mice spent significantly more time near food at night ($F1,22)=394.3$, $p=1.6e^{-15}$) and also that the mice spent more time near food in the post-washout testing, compared to post exposure ($F(1,22)=5.8$, $p=0.024$). For time in thigmotaxis, near water, and in shelter, main effects of time of day were significant, with no interactions; mice were in thigmotaxis and near water more at night, and in their shelters more during the day ($F(1,22)=113.0$, $p=3.9e^{-10}$, $F(1,22)=48.1$, $p=5.8e^{-7}$, and $F(1,22)=12.12$, $p=0.0021$, respectively). No variables or interactions were significantly associated with time in open field.

## 4.5. Discussion

### *4.5.1. Age-Associated Susceptibility to Chronic Domoic Acid Toxicity*

The aged mice in our study showed surprisingly high rates of mortality during chronic exposure to low levels of domoic acid. The rates were so high that we were not able to analyze many of our target endpoints. While limiting our ability to parse the relationship between age and chronic DA exposure now, this underscores the need to further study advanced age as a factor in sensitivity to chronic DA health effects. The consistency with previous research indicating that advanced age also enhances susceptibility to acute DA toxicosis (see Chapter 3 and [27]) further emphasizes this point.

### *4.5.2. Chronic, Low-Level Domoic Acid Exposure and Cardiac Dysfunction*

Cardiac function in adult mice is subtly impacted by repeated, low-level DA exposure before frank toxicity is evident. Our data suggest that repeated, low-level DA exposure in adult mice phenocopies diastolic dysfunction associated with normal aging, and that this dysfunction is persistent through periods without any active DA exposure: the Ea/Aa ratios measured for our DA-exposed animals resembled the Ea/Aa ratios in unexposed mice almost twice their age, and did not improve with washout.

Though early clinicians described arrythmia in humans suffering DA poisoning [6], the majority of laboratory DA research has focused on neurological endpoints. However, reports from environmentally exposed marine mammals corroborate our observations of possible cardiac pathologies from DA. Necropsies of California sea lions (CSLs) with histories of exposure have identified a DA-associated, potentially lethal degenerative cardiomyopathy [31], and cardiac lesions, myofiber necrosis, edema, and nuclear abnormalities are regularly reported in CSL heart

tissue following DA exposure [31–33]. Furthermore, a 2005 study of southern sea otters calculated an odds ratio of 10.6 for dilated cardiomyopathy following suspected DA exposure [34], and a recent longitudinal study of 186 free-ranging sea otters from 2001-2017 found a strong association between chronic environmental DA exposure (approximated using Bayesian spatiotemporal models) and fatal cardiac disease [35]. Interestingly, in the latter study DA exposure was found to be associated with a greater risk for cardiomyopathy in prime-age adults, as opposed to otters of more advanced age. Though more research is needed, the prevailing hypothesis is that in cases where cardiac dysfunction contributes to morbidity or mortality, toxicity results from conduction disturbances and apoptotic pathway activation following DA's direct interaction with cardiac glutamate receptors, not a centrally mediated brain-heart etiology [31].

Moving forward, DA research should include assessments of peripheral organ system effects, in addition to neurological ones, as health effects from chronic DA exposure may manifest through more pathways than those most famously associated with acute exposure. Future work should also consider investigating overt structural damage in the heart, as this could explain the persistence of observed cardiac dysfunction through washout. Research might focus in particular on the left ventricle, as this chamber most directly impacts the diastolic function in which we saw impairment.

### *4.5.3. Chronic, Low-Level Domoic Acid Exposure and Neurological and Behavioral Changes*

The heightened activity levels we saw in adult mice chronically exposed to DA and subsequently allowed a washout period suggest a mild persistent effect of low-dose DA on neurological function. Three of our four activity endpoints were higher in mice exposed to DA,

overall and/or during particular times of day. Interestingly, effects observed after DA exposure were not significantly different from those observed after the washout period. The longer observation period and continuous activity monitoring in this study allowed us to identify the persistence of more subtle neurobehavior endpoints than previously reported memory deficits associated with chronic, low-level DA exposure that were rescued following washout [26]. Thus, the data from this report indicate that the more subtle effects on activity we observed do not appear to be reversible with periods of no exposure.

Mechanisms of chronic DA toxicity and their distinction from acute toxicity mechanisms remain unclear. A 2018 study that administered the antioxidant quercetin to male mice given subconvulsive 2.0 mg/kg DA IP daily for four weeks reported quercetin-associated protection against DA-associated memory impairment, suggesting that oxidative stress is involved [36]. Alternatively, a 2018 study of female mice given 0.75 mg/kg bw DA IP weekly for 22 weeks reported that vesicular glutamate transporter 1 levels and immunoreactivity were increased, possibly pointing to impacts of chronic DA exposure on glutamatergic transmission [37]. Finally, female macaques orally administered 0.075 or 0.15 mg/kg DA/day for up to 11 months and showing symptoms of intention tremors were seen to have changes in hippocampal white matter structure (MRI), increased lactate in the thalamus (MRS), and EEG power differences [38], which might implicate gross structural or electrophysiological changes in the brain [18,38,39], though their functional impact is unclear.

#### *4.5.4. Conclusions and Implications for Human Communities*

A series of studies with the CoASTAL cohort have reported associations between regular consumption of razor clams, a known DA vector, and poorer performance on certain memory

tests [19–21]. Researcher-generated dose-response models estimate that memory impacts may manifest with repeated exposure to DA at levels that do not evoke overt symptoms of toxicity (e.g., GI distress, seizures) and are below current regulatory limits [20]. Our findings are consistent with these reports: we see persistent impacts of chronic exposure to low levels of DA on cardiac function and activity levels in our mouse model. Additionally, CoASTAL studies have repeatedly acknowledged the likelihood that elderly individuals represent a subpopulation in which cognitive impacts may be even greater [19,20]. This is noteworthy, given the severe effects of chronic DA exposure that we observed in our aged mice specifically. If we are to adequately protect public health, it is imperative that we further investigate the health effects of chronic exposure to subconvulsive levels of DA, with particular focus on age-associated susceptibility and both cardiac and neurological injuries.

### 4.6. Acknowledgements

This work was a collaboration between many parties to whom I'm deeply grateful. First, Sophia Liu of the UW Translational Bioenergetics Lab was indispensable. Thank you also to Toby Cole and the UW Animal Behavior Core, without whom this would not have happened. Additional thanks to many members of the Marcinek lab, particularly Rudy Stuppard, Matt Campbell, Ana Valencia, Gavin Pharaoh, Ethan Ostrom, and Sricharan Kannan, and to Megumi Matsushita and Jackie Garrick for assistance with study design. Thank you also to the staff and animals of the UW Animal Research and Care Facilities and UW Veterinary Services, the UW In Vitro Services Core, and the UW Center for Cardiovascular Biology. Finally, deepest gratitude to Dave Marcinek, Kathi Lefebvre, and Tom Burbacher.

## 4.7. Funding

This research was funded by the UW Department of Environmental and Occupational Health Sciences, the NOAA Northwest Fisheries Science Center, National Institutes of Health (NIH) R01s ES021930 and ES030319, National Science Foundation (NSF) grants OCE-1314088 and OCE-183904, and the UW NIEHS sponsored Environmental Pathology/Toxicology Training Program (EP/T) Training Grant (NIEHS T32ES007032).

## 4.8. References

1. Lefebvre, K.A.; Bargu, S.; Kieckhefer, T.; Silver, M.W. From Sanddabs to Blue Whales: The Pervasiveness of Domoic Acid. *Toxicon* **2002**, *40*, 971–977, doi:10.1016/S0041-0101(02)00093-4.
2. Bargu, S.; Powell, C.L.; Coale, S.L.; Busman, M.; Doucette, G.J.; Silver, M.W. Krill: A Potential Vector for Domoic Acid in Marine Food Webs. *Mar Ecol Prog Ser* **2002**, *237*, 209–216, doi:10.3354/MEPS237209.
3. Scholin, C.A.; Gulland, F.; Doucette, G.J.; Benson, S.; Busman, M.; Chavez, F.P.; Cordaro, J.; DeLong, R.; de Vogelaere, A.; Harvey, J.; et al. Mortality of Sea Lions along the Central California Coast Linked to a Toxic Diatom Bloom. *Nature* **2000**, *403*, 80–84, doi:10.1038/47481.
4. White, A.W. Marine Zooplankton Can Accumulate and Retain Dinoflagellate Toxins and Cause Fish Kills. *Limnol Oceanogr* **1981**, *26*, 103–109, doi:10.4319/lo.1981.26.1.0103.
5. White, A.W. Recurrence of Kills of Atlantic Herring (Clupea Harengus Harengus) Caused by Dinoflagellate Toxins Transferred Through Herbivorous Zooplankton. *Canadian Journal of Fisheries and Aquatic Sciences* **1980**, *37*, 2262–2265, doi:10.1139/f80-271.
6. Perl, T.M.; Bédard, L.; Kosatsky, T.; Hockin, J.C.; Todd, E.C.D.; Remis, R.S. An Outbreak of Toxic Encephalopathy Caused by Eating Mussels Contaminated with Domoic Acid. *New England Journal of Medicine* **1990**, *322*, 1775–1780, doi:10.1056/NEJM199006213222504.
7. Todd, E.C.D. Domoic Acid and Amnesic Shellfish Poisoning - A Review. *J Food Prot* **1993**, *56*, 69–83, doi:10.4315/0362-028x-56.1.69.
8. Farmery, A.K.; Alexander, K.; Anderson, K.; Blanchard, J.L.; Carter, C.G.; Evans, K.; Fischer, M.; Fleming, A.; Frusher, S.; Fulton, E.A.; et al. Food for All: Designing Sustainable and Secure Future Seafood Systems. *Rev Fish Biol Fish* **2021**, 1–21, doi:10.1007/S11160-021-09663-X/TABLES/2.
9. Lefebvre, K.A.; Quakenbush, L.; Frame, E.; Huntington, K.B.; Sheffield, G.; Stimmelmayr, R.; Bryan, A.; Kendrick, P.; Ziel, H.; Goldstein, T.; et al. Prevalence of Algal Toxins in Alaskan Marine Mammals Foraging in a Changing Arctic and Subarctic Environment. *Harmful Algae* **2016**, *55*, 13–24, doi:10.1016/j.hal.2016.01.007.

10. Sellner, K.G.; Doucette, G.J.; Kirkpatrick, G.J. Harmful Algal Blooms: Causes, Impacts and Detection. *J Ind Microbiol Biotechnol* **2003**, *30*, 383–406, doi:10.1007/S10295-003-0074-9.
11. Epstein, P.R.; Chivian, E.; Frith, K. Nature's Services: Societal Dependence on Natural Ecosystems. *Plant Health Prog* **2003**, 10, doi:10.1094/PHP.
12. Young, N.; Sharpe, R.A.; Barciela, R.; Nichols, G.; Davidson, K.; Berdalet, E.; Fleming, L.E. Marine Harmful Algal Blooms and Human Health: A Systematic Scoping Review. *Harmful Algae* **2020**, *98*, 101901, doi:10.1016/j.hal.2020.101901.
13. Lefebvre, K.A.; Robertson, A. Domoic Acid and Human Exposure Risks: A Review. *Toxicon* **2010**, *56*, 218–230, doi:10.1016/J.TOXICON.2009.05.034.
14. Hendrix, A.M.; Lefebvre, K.A.; Quakenbush, L.; Bryan, A.; Stimmelmayr, R.; Sheffield, G.; Wisswaesser, G.; Willis, M.L.; Bowers, E.K.; Kendrick, P.; et al. Ice Seals as Sentinels for Algal Toxin Presence in the Pacific Arctic and Subarctic Marine Ecosystems. *Mar Mamm Sci* **2021**, *37*, 1292–1308, doi:10.1111/MMS.12822.
15. Bank, M.S.; Metian, M.; Swarzenski, P.W. Defining Seafood Safety in the Anthropocene. *Environ Sci Technol* **2020**, *54*, 8506–8508, doi:10.1021/ACS.EST.0C03505.
16. Balmer-Hanchey, E.L.; Jaykus, L.A.; Jaykus, L.A.; McClellan-Green, P. Marine Biotoxins of Algal Origin and Seafood Safety. *Journal of Aquatic Food Product Technology* **2008**, *12*, 29–53, doi:10.1300/J030V12N01_03.
17. James, K.J.; Carey, B.; O'Halloran, J.; van Pelt, F.N.A.M.; Škrabáková, Z. Shellfish Toxicity: Human Health Implications of Marine Algal Toxins. *Epidemiol Infect* **2010**, *138*, 927–940, doi:10.1017/S0950268810000853.
18. Petroff, R.; Hendrix, A.; Shum, S.; Grant, K.S.; Lefebvre, K.A.; Burbacher, T.M. Public Health Risks Associated with Chronic, Low-Level Domoic Acid Exposure: A Review of the Evidence. *Pharmacol Ther* **2021**, *227*, doi:10.1016/J.PHARMTHERA.2021.107865.
19. Grattan, L.M.; Boushey, C.; Tracy, K.; Trainer, V.L.; Roberts, S.M.; Schluterman, N.; Morris, J.G. The Association between Razor Clam Consumption and Memory in the CoASTAL Cohort. *Harmful Algae* **2016**, *57*, 20–25, doi:10.1016/j.hal.2016.03.011.
20. Stuchal, L.D.; Grattan, L.M.; Portier, K.M.; Kilmon, K.A.; Manahan, L.M.; Roberts, S.M.; Morris, J.G. Dose-Response Assessment for Impaired Memory from Chronic Exposure to Domoic Acid among Native American Consumers of Razor Clams. *Regulatory Toxicology and Pharmacology* **2020**, *117*, 104759, doi:10.1016/j.yrtph.2020.104759.
21. Grattan, L.M.; Boushey, C.J.; Liang, Y.; Lefebvre, K.A.; Castellon, L.J.; Roberts, K.A.; Toben, A.C.; Morris, J.G. Repeated Dietary Exposure to Low Levels of Domoic Acid and Problems with Everyday Memory: Research to Public Health Outreach. *Toxins (Basel)* **2018**, *10*, doi:10.3390/toxins10030103.
22. Clayton, E.C.; Peng, Y.G.; Means, L.W.; Ramsdell, J.S. Working Memory Deficits Induced by Single but Not Repeated Exposures to Domoic Acid. *Toxicon* **1999**, *37*, 1025–1039, doi:10.1016/S0041-0101(98)00230-X.
23. Basso, D.M.; Beattie, M.S.; Bresnahan, J.C. A Sensitive and Reliable Locomotor Rating Scale for Open Field Testing in Rats. *https://home.liebertpub.com/neu* **2009**, *12*, 1–21, doi:10.1089/NEU.1995.12.1.
24. Xu, R.; Tao, Y.; Wu, C.; Yi, J.; Yang, Y.; Yang, R.; Hong, D. Domoic Acid Induced Spinal Cord Lesions in Adult Mice: Evidence for the Possible Molecular Pathways of Excitatory Amino Acids in Spinal Cord Lesions. *Neurotoxicology* **2008**, *29*, 700–707, doi:10.1016/J.NEURO.2008.04.011.

25. Tiedeken, J.A.; Ramsdell, J.S. Persistent Neurological Damage Associated With Spontaneous Recurrent Seizures and Atypical Aggressive Behavior of Domoic Acid Epileptic Disease. *Toxicological Sciences* **2013**, *133*, 133–143, doi:10.1093/TOXSCI/KFT037.
26. Lefebvre, K.A.; Kendrick, P.S.; Ladiges, W.; Hiolski, E.M.; Ferriss, B.E.; Smith, D.R.; Marcinek, D.J. Chronic Low-Level Exposure to the Common Seafood Toxin Domoic Acid Causes Cognitive Deficits in Mice. *Harmful Algae* **2017**, *64*, 20–29, doi:10.1016/j.hal.2017.03.003.
27. Hesp, B.R.; Clarkson, A.N.; Sawant, P.M.; Kerr, D.S. Domoic Acid Preconditioning and Seizure Induction in Young and Aged Rats. *Epilepsy Res* **2007**, *76*, 103–112, doi:10.1016/j.eplepsyres.2007.07.003.
28. Ferriss, B.E.; Marcinek, D.J.; Ayres, D.; Borchert, J.; Lefebvre, K.A. Acute and Chronic Dietary Exposure to Domoic Acid in Recreational Harvesters: A Survey of Shellfish Consumption Behavior. *Environ Int* **2017**, *101*, 70–79, doi:10.1016/j.envint.2017.01.006.
29. Zheng, G.; Wu, H.; Che, H.; Li, X.; Zhang, Z.; Peng, J.; Guo, M.; Tan, Z. Residue Analysis and Assessment of the Risk of Dietary Exposure to Domoic Acid in Shellfish from the Coastal Areas of China. *Toxins 2022, Vol. 14, Page 862* **2022**, *14*, 862, doi:10.3390/TOXINS14120862.
30. Chiao, Y.A.; Zhang, H.; Sweetwyne, M.; Whitson, J.; Ting, Y.S.; Basisty, N.; Pino, L.K.; Quarles, E.; Nguyen, N.H.; Campbell, M.D.; et al. Late-Life Restoration of Mitochondrial Function Reverses Cardiac Dysfunction in Old Mice. *Elife* **2020**, *9*, 1–26, doi:10.7554/ELIFE.55513.
31. Zabka, T.S.; Goldstein, T.; Cross, C.; Mueller, R.W.; Kreuder-Johnson, C.; Gill, S.; Gulland, F.M.D. Characterization of a Degenerative Cardiomyopathy Associated with Domoic Acid Toxicity in California Sea Lions (Zalophus Californianus). *Vet Pathol* **2009**, *46*, 105–119, doi:10.1354/vp.46-1-105.
32. Gulland, F. *Domoic Acid Toxicity in California Sea Lions (Zalophus Californianus) Stranded Along the Central California Coast, May-October 1998*; NOAA Technical Memorandum NMFS-OPR-17, 2000;
33. Silvagni, P.A.; Lowenstine, L.J.; Spraker, T.; Lipscomb, T.P.; Gulland, F. Pathology of Domoic Acid Toxicity in California Sea Lions (Zalophus Californianus). *Vet Pathol* **2005**, *42*, 184–191, doi:10.1354/vp.42-2-184.
34. Kreuder, C.; Miller, M.A.; Lowenstine, L.J.; Conrad, P.A.; Carpenter, T.E.; Jessup, D.A.; Mazet, J.A.K. Evaluation of Cardiac Lesions and Risk Factors Associated with Myocarditis and Dilated Cardiomyopathy in Southern Sea Otters (Enhydra Lutris Nereis). *Am J Vet Res* **2005**, *66*, 289–299, doi:10.2460/ajvr.2005.66.289.
35. Moriarty, M.E.; Miller, M.A.; Murray, M.J.; Duignan, P.J.; Gunther-Harrington, C.T.; Field, C.L.; Adams, L.M.; Schmitt, T.L.; Johnson, C.K. Exploration of Serum Cardiac Troponin I as a Biomarker of Cardiomyopathy in Southern Sea Otters (Enhydra Lutris Nereis). *Am J Vet Res* **2021**, *82*, 529–537, doi:10.2460/AJVR.82.7.529.
36. Wang, D.; Zhao, J.; Li, S.; Shen, G.; Hu, S. Quercetin Attenuates Domoic Acid-Induced Cognitive Deficits in Mice. *Nutr Neurosci* **2018**, *21*, 123–131, doi:10.1080/1028415X.2016.1231438.
37. Moyer, C.E.; Hiolski, E.M.; Marcinek, D.J.; Lefebvre, K.A.; Smith, D.R.; Zuo, Y. Repeated Low Level Domoic Acid Exposure Increases CA1 VGluT1 Levels, but Not Bouton Density,

VGluT2 or VGAT Levels in the Hippocampus of Adult Mice. *Harmful Algae* **2018**, *79*, 74–86, doi:10.1016/j.hal.2018.08.008.
38. Petroff, R.; Richards, T.; Crouthamel, B.; McKain, N.; Stanley, C.; Grant, K.S.; Shum, S.; Jing, J.; Isoherranen, N.; Burbacher, T.M. Chronic, Low-Level Oral Exposure to Marine Toxin, Domoic Acid, Alters Whole Brain Morphometry in Nonhuman Primates. *Neurotoxicology* **2019**, *72*, 114–124, doi:10.1016/j.neuro.2019.02.016.
39. Petroff, R.; Murias, M.; Grant, K.S.; Crouthamel, B.; McKain, N.; Shum, S.; Jing, J.; Isoherranen, N.; Burbacher, T.M. Power Spectrum Analysis of EEG in a Translational Nonhuman Primate Model after Chronic Exposure to Low Levels of the Common Marine Neurotoxin, Domoic Acid. *Neurotoxicology* **2020**, *80*, 124–129, doi:10.1016/j.neuro.2020.07.006.

### 4.9. Tables and Figures

**Table 4.1.** Survivorship in adult (13 mo at start of experiment) and aged (24 mo at start of experiment) female C57Bl/6 J mice at specified experimental timepoints: all mice were administered saline (S) or 0.5 mg/kg bw DA (DA) for 13 weeks and were then anaesthetized for echocardiography. Aged mice that survived the procedure were sacrificed after echocardiography, while adult mice were given saline or DA for a 14$^{th}$, final week and then allowed a 10-week washout period.

| | | **Survivorship n (%)** | | |
|---|---|---|---|---|
| | **Starting n** | **Exposure (first 13 weeks)** | **Echocardiography** | **Washout period** |
| Adult S | 13 | 12 (92%) | 12 (92%) | 12 (92%) |
| Adult DA | 12 | 12 (100%) | 12 (100%) | 12 (100%) |
| Aged S | 11 | 9 (82%) | 8 (73%) | N/A |
| Aged DA | 13 | 4 (31%) | 3 (23%) | N/A |

**Table 4.2.** Results of three-way repeated measures ANOVA with DA treatment, time of day (day and night), and test round (post exposure versus post washout) as independent variables, and distance traveled, as monitored in Phenotyper cages over a 66-hour observation period, as a dependent variable. *p<0.05, ***p<0.0005.

| **Analysis** | **F-scores and p-values** |
|---|---|
| **Distance traveled** | |
| Three-way interaction | F(1,22)=2.67, p=0.12 |
| Two-way interactions | |
| Treatment x time of day | F(1,22)= 2.84, p=0.11 |
| Treatment x test round | F(1,22)=1.68, p=0.21 |
| Time of day x test round | F(1,22)=0.60, p=0.45 |
| Main effects | |
| Treatment | F(1,22)=4.4, p=0.048 * |
| Time of day | F(1,22)=201.2, $p=1.5e^{-12}$ *** |
| Test round | F(1,22)= 0.22, p=0.64 |

**Table 4.3.** Results of three-way repeated measures ANOVAs with DA treatment, time of day (day and night), and test round (post exposure versus post washout) as independent variables, and time spent in three discrete states of mobility – high mobility, mobility, and immobility – over the course of a 66-hour observation period in Phenotyper cages as dependent variables in turn. *p<0.05, **p<0.005, ***p<0.0005.

| **Analysis** | **F-scores and p-values** |
|---|---|
| **High Mobility** | |
| Three-way interaction | F(1,22)=2.01, p=0.17 |
| Two-way interactions | |
| Treatment x time of day | F(1,22)=10.2, p=0.0042 ** |
| Treatment x test round | F(1,22)=1.04, p=0.32 |
| Time of day x test round | F(1,22)=3.11, p=0.092 |
| Simple effects | |
| Treatment during the day | F(1,22)=9.1, p=0.0064 ** |
| Treatment during the night | F(1,22)=11.0, p=0.0031*** |
| **Mobility** | |
| Three-way interaction | F(1,22)=2.02, p=0.17 |
| Two-way interactions | |
| Treatment x time of day | F(1,22)=4.10, p=0.055 |
| Treatment x test round | F(1,22)=1.99, p=0.17 |
| Time of day x test round | F(1,22)=1.0, p=0.33 |
| Main effects | |
| Treatment | F(1,22)=5.38, p=0.03 * |
| Time of day | F(1,22)=68.92, $p=3.2e^{-8}$ *** |
| Test round | F(1,22)= 1.25, p=0.28 |
| **Immobility** | |
| Three-way interaction | F(1,22)=1.02, p=0.33 |
| Two-way interactions | |
| Treatment x time of day | F(1,22)=0.26, p=0.61 |
| Treatment x test round | F(1,22)=0.13, p=0.73 |
| Time of day x test round | F(1,22)=1.81, p=0.19 |
| Main effects | |
| Treatment | F(1,22)=1.33, p=0.26 |
| Time of day | F(1,22)=155.73, $p=1.9e^{-11}$ *** |
| Test round | F(1,22)= 0.016, p=0.90 |

**Table 4.4.** Results of three-way repeated measures ANOVAs with DA treatment, time of day (day and night), and test round (post exposure versus post washout) as independent variables and time spent in five regions of Phenotyper cages – open field, thigmotaxis (perimeter), near water, near food, and in shelter – during 66 hours of observation as dependent variables in turn. *p<0.05, **p<0.005, ***p<0.0005.

| **Analysis** | **F-scores and p-values** |
|---|---|
| **Open field** | |
| Three-way interaction | F(1,22)=2.3, p=0.14 |
| Two-way interactions | |
| Treatment x time of day | F(1,22)=0.58, p=0.45 |
| Treatment x test round | F(1,22)=0.0, p=0.99 |
| Time of day x test round | F(1,22)=4.00, p=0.060 |
| Main effects | |
| Treatment | F(1,22)=0.021, p=0.89 |
| Time of day | F(1,22)=0.062, p=0.81 |
| Test round | F(1,22)=4.0, p=0.057 |
| **Thigmotaxis** | |
| Three-way interaction | F(1,22)=3,3, p=0.083 |
| Two-way interactions | |
| Treatment x time of day | F(1,22)=0.036, p=0.85 |
| Treatment x test round | F(1,22)=0.10, p=0.75 |
| Time of day x test round | F(1,22)=0.21, p=0.65 |
| Main effects | |
| Treatment | F(1,22)=0.44, p=0.51 |
| Time of day | F(1,22)=113.04, $p=3.9e^{-10}$ *** |
| Test round | F(1,22)=2.7, p=0.11 |
| **Water** | |
| Three-way interaction | F(1,22)=0.28, p=0.60 |

**Table 4.4.** *continued*

| | |
|---|---|
| Two-way interactions | |
| Treatment x time of day | F(1,22)=0.44, p=0.52 |
| Treatment x test round | F(1,22)=0.66, p=0.43 |
| Time of day x test round | F(1,22)=2.4, p=0.13 |
| Main effects | |
| Treatment | F(1,22)=2.17, p=0.16 |
| Time of day | F(1,22)=48.1, p=5.8e$^{-7}$ *** |
| Test round | F(1,22)= 3.1, p=0.092 |
| **Food** | |
| Three-way interaction | F(1,22)=1.26, p=0.27 |
| Two-way interactions | |
| Treatment x time of day | F(1,22)=0.12, p=0.73 |
| Treatment x test round | F(1,22)=0.21, p=0.65 |
| Time of day x test round | F(1,22)=2.8, p=0.11 |
| Main effects | |
| Treatment | F(1,22)=0.042, p=0.84 |
| Time of day | F(1,22)=394.3, p=1.2e$^{-15}$ *** |
| Test round | F(1,22)= 5.8, p=0.024 * |
| **Shelter** | |
| Three-way interaction | F(1,22)=1.64, p=0.21 |
| Two-way interactions | |
| Treatment x time of day | F(1,22)=0.47, p=0.50 |
| Treatment x test round | F(1,22)=0.0, p=1.0 |
| Time of day x test round | F(1,22)=0.50, p=0.49 |
| Main effects | |
| Treatment | F(1,22)=0.88, p=0.34 |
| Time of day | F(1,22)=12.12, p=0.0021 ** |
| Test round | F(1,22)= 0.50, p=0.49 |

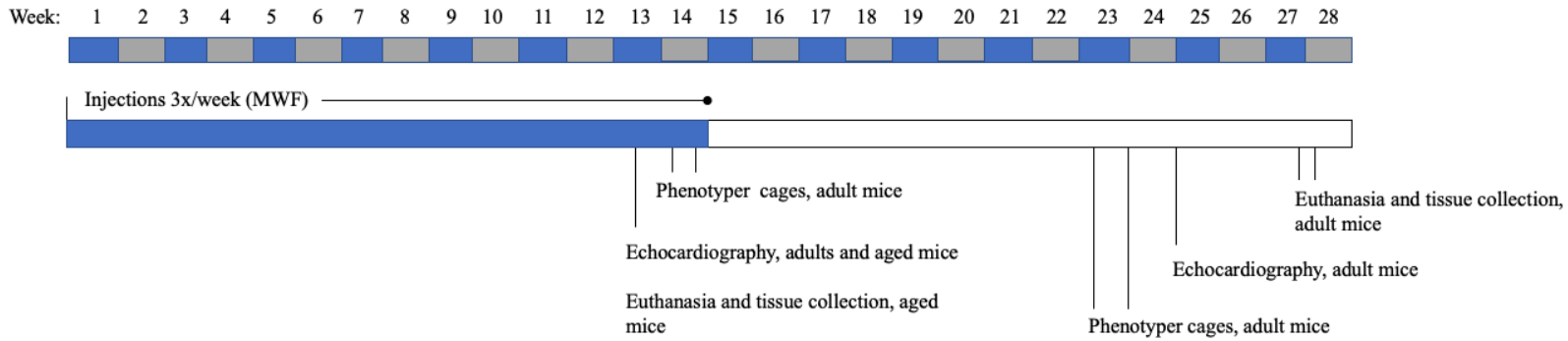


**Figure 4.1.** Schematic of study design lasting 28 weeks. Adult and aged female C57Bl/6 J mice were IP injected with 0.5 mg/kg bw DA three times each week for 14 weeks, and then allowed a 10-week washout period. Echocardiography was performed on aged control animals and control and DA-exposed adult animals one week before the end of the exposure period (i.e., in the 13$^{th}$ week) and then aged animals were sacrificed. Adult mice were monitored in Phenotyper activity cages at the end of the exposure period (i.e., in the 14$^{th}$ week), and then echocardiography and activity monitoring were performed on adult mice again at the end of the washout period, before they were sacrificed.

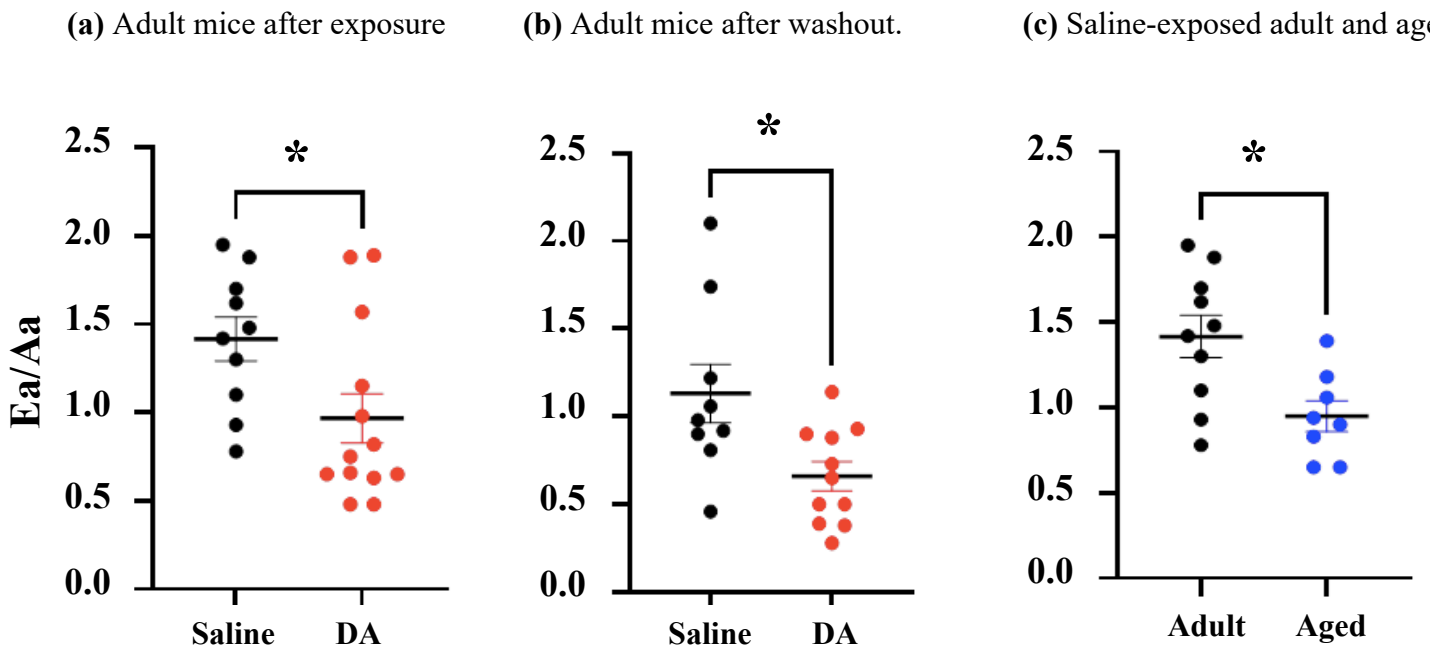


**Figure 4.2.** Diastolic function of (a) adult female C57Bl/6 J mice after being administered either saline or 0.5 mg/kg bw DA IP for 13 weeks and then (b) given one additional week of saline or DA administration and then allowed 10 weeks of depuration, and (c) adult and aged female C57Bl/6 J mice administered saline for 13 weeks. T-test, *$p \leq 0.05$. *All data in this figure were collected and analyzed by Sophia Liu of the UW Translational Bioenergetics Lab.*

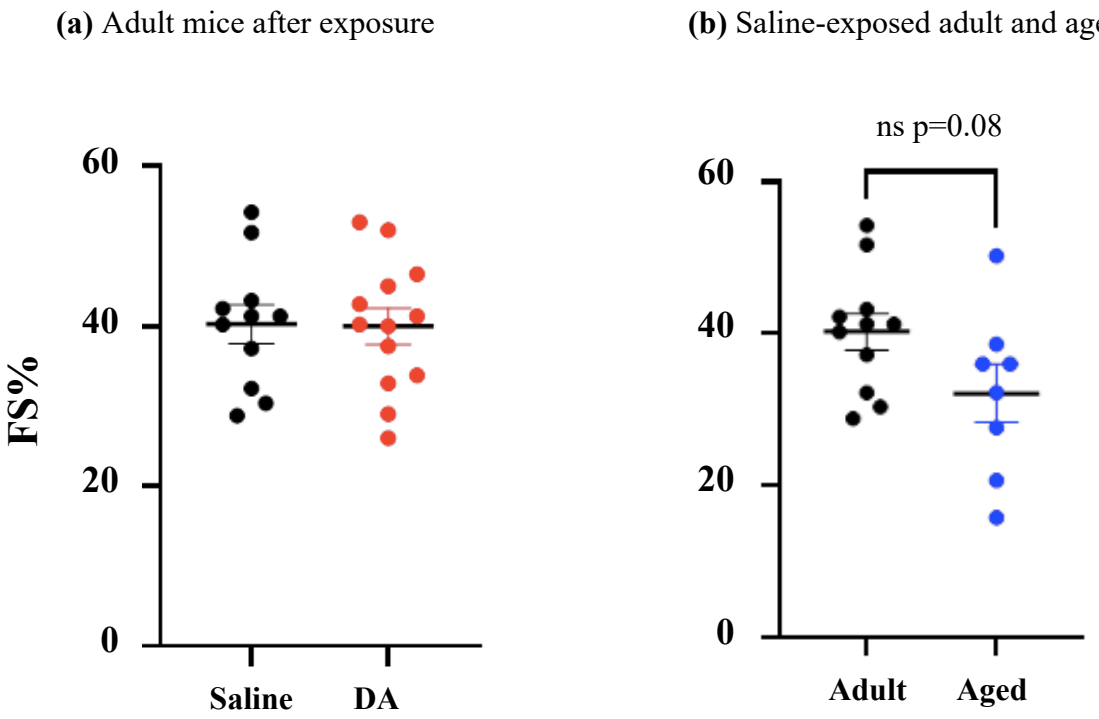


**Figure 4.3.** Systolic function of (a) adult female C57Bl/6 J mice administered either saline or 0.5 mg/kg bw DA IP for 13 weeks, and (b) adult and aged C57Bl/6 J female mice administered saline for 13 weeks. T-test, $p \geq 0.05$.

*All data in this figure were collected and analyzed by Sophia Liu of the UW Translational Bioenergetics Lab.*

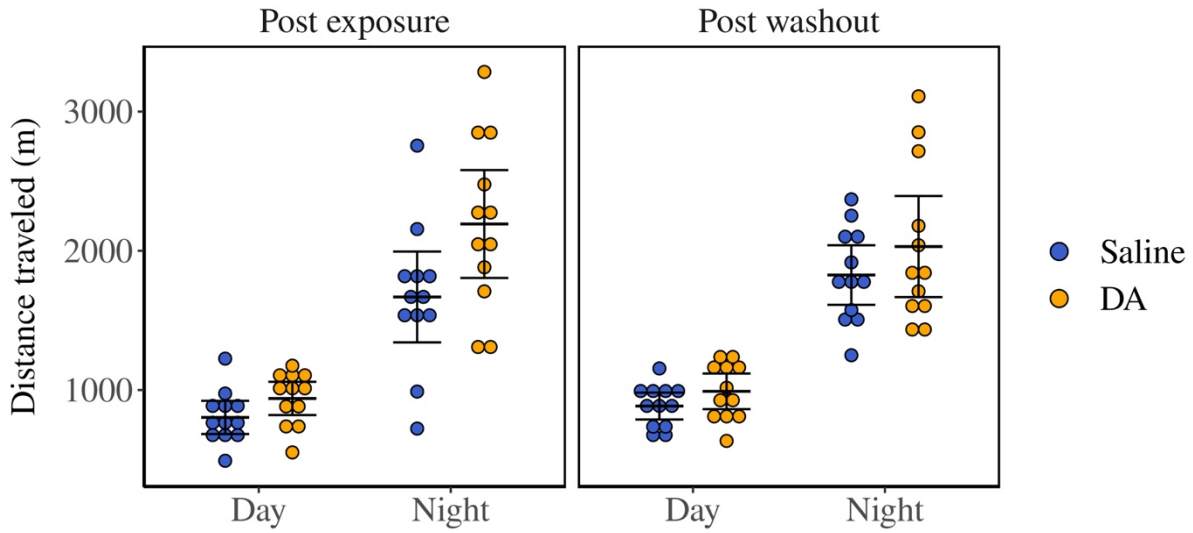


**Figure 4.4.** Distances traveled by adult female C57Bl/6 J mice given saline (blue) or 0.5 mg/kg bw DA (orange) thrice weekly for 14 weeks, as monitored in Phenotyper activity cages over 66 hours. Binned data are shown for all daylight hours and all nighttime hours.

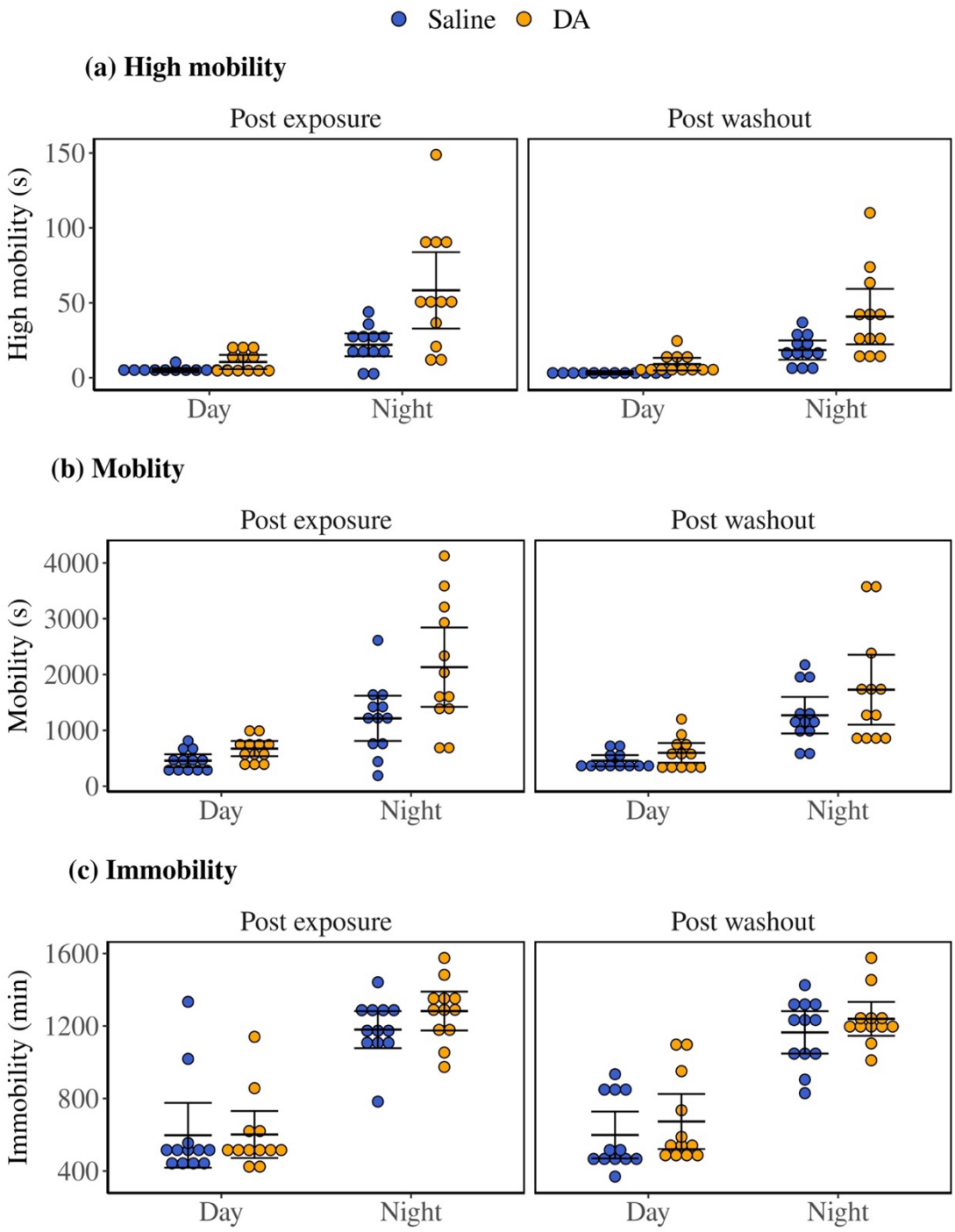


**Figure 4.5.** Time adult female C57Bl/6 J mice given saline (blue) or 0.5 mg/kg bw DA (orange) thrice weekly for 14 weeks spent in each of three discrete levels of mobility – high mobility (a), mobility (b), and immobility (c), when monitored in Phenotyper activity cages over 66 hours. Binned data are shown for all daylight hours and all nighttime hours.

## Chapter 5: Conclusions and Future Directions

### 5.1. Dissertation Summary

The algal-produced neurotoxin domoic acid (DA) has posed a known threat to wildlife and human public health since its recognition following a human poisoning event in 1987 [1]. Though regulatory limits protect seafood consumers from lethal doses [2,3], shifting ocean conditions could mean changing threats for coastal communities and seafood consumers. Primary areas for research include changing regions of concern for toxic contamination, interindividual susceptibility factors, and health effects of emerging exposure scenarios.

In our first aim, we used samples from Alaskan ice seals to assess the extent to which DA may be spreading into new regions. Though historically DA has primarily been a concern for wildlife and humans on the western coast of the contiguous United States, ocean warming in the past decades means that conditions favorable to algae growth may spread to regions previously too cold, like the Arctic and subarctic [4–8]. To assess the possibility of broadening DA contamination, we analyzed hundreds of samples collected from the gastrointestinal tracts of four species of ice seal in the Bering, Chukchi, and Beaufort seas around Alaska for presence and levels of DA. Not only did we find DA in samples from all three seas and all four seal species, we also found a significant increase in DA prevalence in stomach content samples from bearded seals in the Bering Sea over time [9]. We additionally drew conclusions that benthic prey pose particular risk for toxin exposure, and that colon content samples consistently contain higher toxin levels than stomach content samples, two pieces of information that will help direct ongoing monitoring programs in the Alaskan region.

In aim 2 we followed up on suggestions that both male sex and advanced age are associated with increased DA sensitivity [1,10,11]. We observed seizure-related activity

following one-time administration of DA doses ranging from 0.5 to 2.5 mg/kg bw in female and male adult and aged mice, and collected tissues 90 minutes after exposure, in which we quantified persistent DA levels. Full clonic-tonic convulsions only occurred in our aged mice, and occurred at greater rates in females. Survival analysis additionally indicated that age and female sex were associated with incidence of hindlimb tremors, a less severe but well-documented DA symptom. Finally, we saw higher concentrations of DA in tissues from aged animals as compared to adults, and from female mice as compared to male counterparts.

Our final experiments, in aim 3, focused on exposure paradigms receiving increasing attention from the DA researcher community, namely those in which consumers repeatedly or chronically ingest low levels of DA [12]. In this study, we administered 0.5 mg/kg bw DA to female adult and aged mice thrice weekly for a total of 14 weeks. During exposure, we observed remarkably high rates of DA-associated mortality in our aged females. However, we were able to measure cardiac function via echocardiography in our aged control animals and our control and DA-exposed adults. These tests indicated a DA-related diastolic cardiac dysfunction phenotype similar to that associated with aging. In our adult mice we were also able to assess activity- and anxiety-related behaviors at the end of the exposure period and then again after washout. We found consistent evidence that our DA exposure regimen was associated with heightened levels of activity, and that these effects were not mitigated by washout.

### 5.2. Conclusions

The body of work presented in this dissertation explores the growth and transformation of public health threats presented by DA. We outline data indicating significant increases in DA contamination of Bering Sea food webs. We show that older females may be especially at risk of

injury from more ubiquitous DA production. We describe neurological and cardiac functional changes associated with repeated exposure to low levels of DA and suggest that advanced age may again exacerbate toxicity from levels below those conventionally understood to present health risks. Collectively, these findings will help us to take appropriate protective actions to safeguard both our wildlife and human communities.

### 5.3. Future Directions

This dissertation addresses three primary knowledge gaps in the DA literature and in so doing identifies key directions for future research. First, in documenting an apparent increasing risk of DA exposure in Alaskan regions, we have highlighted the importance of continuing and even expanding monitoring programs in the region to protect public health. Such programs can consider targeting colon content samples from primarily benthic feeders to maximize effectiveness [9]. Second, our findings that female mice, particularly of more advanced age, appear to be more sensitive to acute DA injury require additional laboratory investigation, as this is contrary to previous DA work which proposed greater susceptibility in males [1,11]. In such future studies researchers should consider the role of sex hormones and their fluctuations over the lifespan; the loss of sex hormone-associated protection against excitotoxicity may contribute to the severe symptoms observed in older female mice [11,13,14]. The observation that aged females experienced high mortality following chronic DA exposure further supports the need to include assessment of age-related susceptibility in DA research moving forward. Finally, while some repeated, low-level DA dosing regimens have been connected to reversible memory effects [15], the regimen described in our experiments results in behavioral changes that persist through a depuration period almost as long as the initial exposure. More work will clarify the

mechanisms by which such subtle behavioral pathologies emerge and persist beyond direct exposure periods. Our findings also show that ongoing research into chronic DA exposure scenarios should assess endpoints in more diverse tissue systems, particularly cardiac function, beyond the current focus on neurological function [12].

### 5.4. References


1. Perl, T.M.; Bédard, L.; Kosatsky, T.; Hockin, J.C.; Todd, E.C.D.; Remis, R.S. An Outbreak of Toxic Encephalopathy Caused by Eating Mussels Contaminated with Domoic Acid. *New England Journal of Medicine* **1990**, *322*, 1775–1780, doi:10.1056/NEJM199006213222504.
2. Wekell, J.C.; Hurst, J.; Lefebvre, K.A. The Origin of the Regulatory Limits for PSP and ASP Toxins in Shellfish. *J Shellfish Res* **2004**, *23*, 927–930.
3. Mariën, K. Establishing Tolerable Dungeness Crab (Cancer Magister) and Razor Clam (Siliqua Patula) Domoic Acid Contaminant Levels. *Environ Health Perspect* **1996**, *104*, 1230, doi:10.1289/EHP.104-1469507.
4. Ekstrom, J.A.; Moore, S.K.; Klinger, T. Examining Harmful Algal Blooms through a Disaster Risk Management Lens: A Case Study of the 2015 U.S. West Coast Domoic Acid Event. *Harmful Algae* **2020**, *94*, 101740, doi:10.1016/J.HAL.2020.101740.
5. McCabe, R.M.; Hickey, B.M.; Kudela, R.M.; Lefebvre, K.A.; Adams, N.G.; Bill, B.D.; Gulland, F.; Thomson, R.E.; Cochlan, W.P.; Trainer, V.L. An Unprecedented Coastwide Toxic Algal Bloom Linked to Anomalous Ocean Conditions. *Geophys Res Lett* **2016**, *43*, 10,366-10,376, doi:10.1002/2016GL070023.
6. Anderson, D.M.; Richlen, M.L.; Lefebvre, K.A. Harmful Algal Blooms in the Arctic. In *Arctic Report Card 2018*; Osborne, E., Richter-Menge, J., Jeffries, M., Eds.; 2018; pp. 81–87.
7. Lefebvre, K.A.; Quakenbush, L.; Frame, E.; Huntington, K.B.; Sheffield, G.; Stimmelmayr, R.; Bryan, A.; Kendrick, P.; Ziel, H.; Goldstein, T.; et al. Prevalence of Algal Toxins in Alaskan Marine Mammals Foraging in a Changing Arctic and Subarctic Environment. *Harmful Algae* **2016**, *55*, 13–24, doi:10.1016/j.hal.2016.01.007.
8. Trainer, V.L.; Cochlan, W.P.; Erickson, A.; Bill, B.D.; Cox, F.H.; Borchert, J.A.; Lefebvre, K.A. Recent Domoic Acid Closures of Shellfish Harvest Areas in Washington State Inland Waterways. *Harmful Algae* **2007**, *6*, 449–459, doi:10.1016/j.hal.2006.12.001.
9. Hendrix, A.M.; Lefebvre, K.A.; Quakenbush, L.; Bryan, A.; Stimmelmayr, R.; Sheffield, G.; Wisswaesser, G.; Willis, M.L.; Bowers, E.K.; Kendrick, P.; et al. Ice Seals as Sentinels for Algal Toxin Presence in the Pacific Arctic and Subarctic Marine Ecosystems. *Mar Mamm Sci* **2021**, *37*, 1292–1308, doi:10.1111/MMS.12822.
10. Hesp, B.R.; Clarkson, A.N.; Sawant, P.M.; Kerr, D.S. Domoic Acid Preconditioning and Seizure Induction in Young and Aged Rats. *Epilepsy Res* **2007**, *76*, 103–112, doi:10.1016/j.eplepsyres.2007.07.003.

11. Baron, A.W.; Rushton, S.P.; Rens, N.; Morris, C.M.; Blain, P.G.; Judge, S.J. Sex Differences in Effects of Low Level Domoic Acid Exposure. *Neurotoxicology* **2013**, *34*, 1–8, doi:10.1016/J.NEURO.2012.10.010.
12. Petroff, R.; Hendrix, A.; Shum, S.; Grant, K.S.; Lefebvre, K.A.; Burbacher, T.M. Public Health Risks Associated with Chronic, Low-Level Domoic Acid Exposure: A Review of the Evidence. *Pharmacol Ther* **2021**, *227*, doi:10.1016/J.PHARMTHERA.2021.107865.
13. Azcoitia, I.; Fernandez-Galaz, C.; Sierra, A.; Garcia-Segura, L.M. Gonadal Hormones Affect Neuronal Vulnerability to Excitotoxin-Induced Degeneration. *Journal of Neurocytology 1999 28:9* **1999**, *28*, 699–710, doi:10.1023/A:1007025219044.
14. Kerr, D.; Razak, A.; Crawford, N. Age-Related Changes in Tolerance to the Marine Algal Excitotoxin Domoic Acid. *Neuropharmacology* **2002**, *43*, 357–366, doi:10.1016/S0028-3908(02)00088-6.
15. Lefebvre, K.A.; Kendrick, P.S.; Ladiges, W.; Hiolski, E.M.; Ferriss, B.E.; Smith, D.R.; Marcinek, D.J. Chronic Low-Level Exposure to the Common Seafood Toxin Domoic Acid Causes Cognitive Deficits in Mice. *Harmful Algae* **2017**, *64*, 20–29, doi:10.1016/j.hal.2017.03.003.