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Rapid behavioural diagnosis of domoic acid toxicosis in California sea lions

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Domoic acid is a neurotoxic metabolite of widely occurring algal blooms that has caused multiple marine animal stranding events. Exposure to high doses of domoic acid, a glutamate agonist, may lead to persistent medial temporal seizures and damage to the hippocampus. California sea lions (*Zalophus californianus*) are among the most visible and frequent mammalian victims of domoic acid poisoning, but rapid, reliable diagnosis in a clinical setting has proved difficult owing to the fast clearance of the toxin from the blood stream. Here, we show that the behavioural orienting responses of stranded sea lions diagnosed with domoic acid toxicosis habituate more slowly to a series of non-aversive auditory stimuli than do those of sea lions with no apparent neurological deficits. A signal detection analysis based on these habituation measures was able to correctly identify 50 per cent of subjects with domoic acid toxicosis while correctly rejecting approximately 93 per cent of controls, suggesting potential diagnostic merit.

Keywords: behavioural diagnostics; habituation; hippocampus; domoic acid; marine mammals; sea lions

1. INTRODUCTION

Over the last decade, marine mammal stranding events coincident with large blooms of *Pseudonitschzia australis* have become increasingly common [1]. The factors producing this increase are complicated, but probably include interactions between marine mammal feeding and migratory patterns, and location and timing of blooms [2]. Some types of *Pseudonitschzia* diatoms produce domoic acid, a glutamate agonist with high affinity for AMPA and kainate receptors [3]. Domoic acid is cleared from the body rapidly [4], but persistent excitotoxic effects frequently result in neuronal degradation, particularly in the hippocampus and surrounding medial temporal region [5]. Such neuronal necrosis is particularly acute in the dentate gyrus and hippocampal sectors CA4, CA3 and CA1 [6,7].

California sea lions have been particularly visible victims of domoic acid exposure and toxicosis. In magnetic resonance images (MRIs) of the brain of 42 sea lions diagnosed with chronic domoic acid toxicosis at The Marine Mammal Center in Sausalito, CA, USA, 41 showed detectable hippocampal atrophy, ranging from mild to severe [1]. Seventy of 89 animals with chronic domoic acid toxicosis that died during the same period exhibited gross hippocampal lesions at necropsy, most commonly in sector CA3 and the dentate gyrus.

Diagnosing domoic acid toxicosis in a clinical setting is generally a haphazard or time-intensive and expensive endeavour [8]. Direct diagnosis from blood sampling is rare as domoic acid is cleared from the blood stream within 48 h [9], and animals are often not accessible for treatment until days after exposure [1]. At The Marine Mammal Center, live animals are typically suspected of domoic acid poisoning on the basis of epidemiology and an initial clinical neurological examination—greater reliability of diagnosis relies on post hoc assessment involving laboratory estimation of domoic acid content of urine or faeces, and analysis of the brain, either by post-mortem histology or MRI. Both electroencephalography (EEG), which identifies patterns of seizures, and MRI, which can identify significant neuronal necrosis and atrophy in the hippocampus, are effective but relatively slow and expensive diagnostic tools, and require sedation. Many cases of domoic acid toxicosis are only accurately diagnosed during post-mortem examination by histological examination of the hippocampus [6]. Diagnosis determines the course of treatment and prognosis, and factors into veterinary decisions to release or euthanize stranded sea lions. Therefore, improved methods for *in vivo* diagnosis are needed.

This first attempt at devising a simple and empirically grounded behavioural diagnostic assay of domoic acid toxicosis depends on the habituation of an orienting response to non-aversive auditory stimuli. As hippocampal necrosis is a common consequence of domoic acid toxicosis in sea lions, and hippocampal damage has been shown to slow habituation rates of unrestricted exploratory behaviour in a range of species [10–12], a metric based on habituation holds diagnostic promise. Further, the habituation of an orienting response can be measured through observation, requiring no invasive or aversive procedures [13]. Here, we present the results of a behavioural assessment designed to be sensitive to hippocampal damage in order to augment diagnosis of domoic acid toxicosis in sea lions.

2. MATERIAL AND METHODS

Forty-two sea lions undergoing rehabilitation at The Marine Mammal Center were sampled in this study (see the electronic supplementary material, subject table). Effort was made to test all available admitted sea lions, regardless of diagnosis, during the study period. After testing was complete, subjects were assessed using veterinary clinical criteria wholly independent of performance in testing. Twelve sea lions were diagnosed with domoic acid toxicosis and 27 sea lions, forthwith referred to as 'controls', were evaluated as having no signs of domoic acid toxicosis or other neurological abnormalities. A positive diagnosis of domoic acid toxicosis was based either on clinical signs of seizures and ataxia that resolved following diuresis and sedation (as described in [6]) *and* presence of domoic acid in urine or faeces; *or* on detection of an abnormal hippocampus through MRI or post-mortem histology. Three sea lions had indeterminate diagnoses and were not included in the final sample.

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The behavioural assessments were conducted in a quiet pen. Following acclimation, each sea lion was exposed to a series of auditory stimuli in four sequential test phases. The testing sequence was designed to examine initial habituation of orienting responses to novel auditory stimuli, and to probe recovery of response, or dishabituation, following manipulations of spatial presentation, recovery interval and stimulus type. Each test phase comprised successive presentations of one of two sounds from one of two diametrically opposite locations (see the electronic supplementary materials for further details on experimental manipulations).

During testing, an experimenter, blind to subject diagnosis, observed subject behaviour and coded responses in real time from closed circuit video. An orienting response emitted following stimulus onset, and within 0.5 s of stimulus offset, was considered a positive response. Orienting was defined as a noticeable change in the angle of the subject's head towards the source of the stimulus in the vertical or horizontal plane. During each of the four testing phases, the auditory stimulus was presented on a fixed, semi-random schedule at intervals of 5–15 s until the subject habituated, at which point the next phase began. 'Habituation' was defined as no observable orientation to three consecutive stimuli. The experimenter's real-time assessment of habituation was used during testing and for subsequent analysis. These scores were later validated by two independent observers who viewed the videotaped recordings of each session.

Exposures to habituation were compared between sea lions with domoic acid toxicosis and controls for each of the four test phases using *t*-tests with Bonferroni corrections for repeated measures. Test phases showing a significant difference between these groups were then further subjected to signal detection analysis employing receiver operating characteristic (ROC) curves. ROC curves assess a metric's likelihood of producing a correct positive diagnosis relative to the likelihood of a false positive diagnosis over a range of diagnostic sensitivity thresholds. Thresholds used here were the number of exposures prior to habituation in a particular test phase.

3. RESULTS

Sea lions with domoic acid toxicosis took significantly more exposures to habituate in the first test phase than did controls (figure 1)—there were no significant differences observed in test phases 2–4. Agreement between the experimenter's initial coding of orienting behaviour across all subjects and exposures and that of the post hoc observers was 85 and 86 per cent.

A ROC curve was computed using independent diagnosis of domoic acid toxicosis and the number of exposures to habituation in phase 1 (figure 2). The area under the curve was 0.82, suggesting a good diagnostic metric.

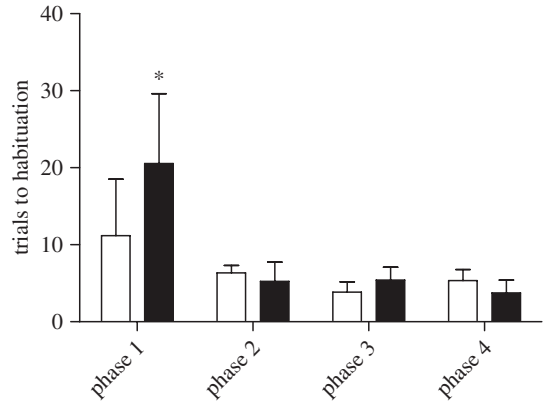

Figure 1. Responsiveness across auditory phases. Mean number of exposures prior to habituation for subjects diagnosed with domoic acid toxicosis and controls in four test phases: initial exposure, exposure following a spatial shift, exposure following a delay and exposure following a stimulus shift. Error bars represent standard deviation. There was a significant difference (asterisk) between exposures to habituation for sea lions with and without domoic acid toxicosis in the first ($p < 0.001$) but not the second, third or fourth test phases ($p > 0.05$, *t*-tests with Bonferroni correction for repeated sampling). White bars, control; black bars, domoic acid.

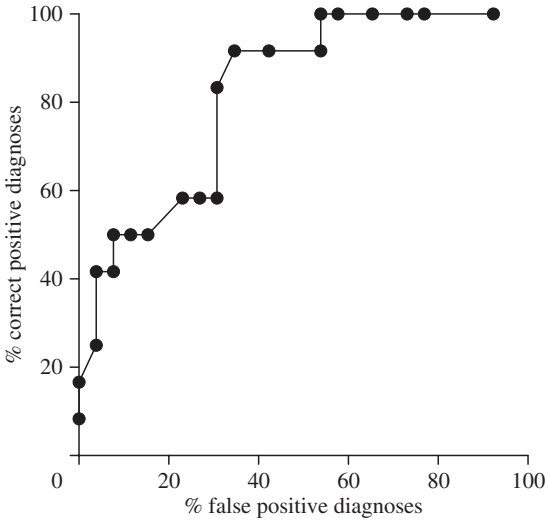

Figure 2. ROC curve based on exposures prior to habituation in test phase 1. This ROC curve represents the diagnostic effectiveness of a metric based on exposures prior to habituation in the first test phase. The likelihood of producing a correct positive diagnosis (represented on the *y*-axis) versus the likelihood of a false positive diagnosis (represented on the *x*-axis) was computed across a range of sensitivity thresholds. These thresholds were defined as the number of exposures prior to habituation above which an animal was considered to have domoic acid toxicosis. For the ROC curve, diagnosis was considered correct if it matched the independent veterinary assessment, and incorrect if it did not. The area under the curve was 0.82 and $p < 0.002$.

4. DISCUSSION

The notable tendency of sea lions with domoic acid toxicosis to habituate more slowly to a non-aversive auditory stimulus may be explained by the presence of hippocampal damage in these subjects. There were no significant differences in responsiveness between subject groups in phases 2–4, suggesting that the spatial, delay and stimulus manipulations did not have differential effects on animals with domoic acid toxicosis. More generally, this result indicates that these dishabituation measures were relatively insensitive to confirmed or presumed hippocampal damage.

Quantitative behavioural diagnostics are rarely used in veterinary clinical settings, but in this case, ROC analysis of exposures to habituation in the first test phase indicates that habituation is a promising measure to augment current diagnostic approaches to domoic acid toxicosis. ROC analysis produces a ratio of correct positive diagnoses to false positive diagnoses across a range of thresholds (see the electronic supplementary material, ROC criteria table). In the case of domoic acid toxicosis in sea lions, false diagnosis of domoic acid toxicosis could lead to an otherwise healthy sea lion's being euthanized (as recommended in [14]), so ultimately, a conservative diagnostic threshold of greater than 22 exposures prior to habituation was

selected for this study. Using this threshold, the habituation measure correctly identified 50 per cent of sea lions with domoic acid toxicosis while falsely diagnosing only 7 per cent of control subjects. This auditory response test can serve as a rapid, inexpensive and logistically easy diagnostic test for hippocampal damage available to most practitioners in the absence of advanced and expensive clinical diagnostics such as MRI or EEG. This represents a novel and applicable behavioural approach to diagnosis of a neurological disorder in a veterinary setting.

Further refinement of the procedure is ongoing in concert with a study of sea lions with domoic acid toxicosis in which all subjects are undergoing MR brain imaging. This may improve an already effective diagnostic measure, and will indicate whether the behavioural assay discussed here is sensitive to hippocampal damage as suspected or to other sequelae of domoic acid toxicosis.

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