Cortisol levels in narwhal (*Monodon monoceros*) blubber from 2000-2019

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Abstract

Narwhals (*Monodon monoceros*) summering on northern Baffin Island are experiencing increases in vessel traffic related to an iron-ore mine operated by Baffinland Iron Mines Corporation; how this increase in vessel traffic may impact narwhal is currently unknown. Cortisol is a stress response hormone and a stress indicator in marine mammals. This study evaluated cortisol levels in narwhal blubber sampled during subsistence harvests prior to project related vessel traffic (2000-2006), during project related vessel traffic (2013-2019), and during a high-stress entrapment event that occurred in 2015. There was a significant increase in cortisol levels from pre- (0.81 ± 0.45 ng/g [±SE]) to during (1.81 ± 0.48 ng/g [±SE]) project related vessel traffic (over 100% higher), and both were significantly lower than cortisol levels from animals sampled during an entrapment event (10.52 ± 0.59 ng/g [±SE]). Increased vessel traffic, changing ice conditions, altered Arctic food webs, increased predation pressure from killer whales, and cumulative impacts from these sources likely all contribute to increased stress levels for narwhals. Thus, there is a need for continued monitoring of stress-responses (i.e. cortisol levels) and other health indicators in narwhals to understand how individual fitness and the population will be impacted over time.

Key words: stress hormones, shipping, vessel traffic, Arctic,

Introduction

Narwhals (*Monodon monoceros*) are part of the annual subsistence hunt in Nunavut, Canada, and are both culturally and economically important to Inuit communities. Increases in anthropogenic carbon in the atmosphere have resulted in increased temperatures and reduced sea ice extent in the Arctic (Serreze and Stroeve 2015). A longer open water season has resulted in increased industrial vessel traffic in the Arctic (Dobson et al. 2018) and this increase is predicted to continue well into the 21st century (Stephenson et al. 2013). Whales living in the Arctic have not experienced these unprecedented levels of vessel traffic and how they will respond to these disturbances is currently unknown. Studies have shown that vessel traffic can alter the movement, behaviour, acoustic and stress response for marine mammals (Rolland et al. 2012; Erbe et al. 2019; Gomez et al. 2016), as well as the movement and behaviour of their prey (Ivanova et al. 2020).

Cortisol is the primary steroid hormone produced in response to perceived stressors (Dobson and Smith 2000). Cortisol acts as an indicator for the stress response in mammals and is positively correlated with the severity of the stressor (Hennessy et al. 1979, Bechshoft et al. 2020). For example, higher stress levels in baleen whales have been correlated with periods of intense commercial whaling (Trumble et al. 2019) and in bottlenose dolphins (*Tursiops truncatus*) cortisol increased significantly during stress experiments where dolphins were trained to voluntarily beach themselves for two hours (Bechshoft et al. 2020). There is also evidence that chronic stress reduces fitness (Dobson and Smith 2000, Wright et al. 2007) likely through suppression of reproduction (Suzuki et al. 2003). Suzuki et al. (2003) found a negative correlation between cortisol and the sex hormones testosterone and progesterone levels in killer whales (*Orcinus orca*) and Indo-Pacific bottlenose dolphins (*Tursiops aduncus*). How short-term stress directly influences reproduction is not well understood, but chronic stress typically results in inhibition of reproduction (Tilbrook et al. 2000). The timescale on which the effects of short versus long-term stressors are observed will vary for different species. Individual stress response may have significant bearing at the population level, particularly if they are of reproductive age.

Baffinland Iron Mines Corporation developed the Mary River Project (mining and shipping of iron ore) that started producing ore in 2015, but began vessel traffic for mine preparation in 2013, on northern Baffin Island (Baffinland 2018). This area is considered a high use area for narwhals from the Eclipse Sound stock (Figure 1), with an estimated population of ~12,000 individuals (Marcoux et al. 2019). Baffinland is proposing Phase 2 of their project to start in 2024 that will increase the transport of iron ore from 6 to 12 Mtpa[3] and the use of icebreaking in the spring and fall. This phase would include an estimated 176 ore carrier round trips, using larger cape-size vessels with deadweight tonnage of 130,000-250,000 tonnes, and ice-breakers vessels (DFO 2020). Project related vessel traffic within the region has been ongoing since 2013, including within the narwhal summering area (Figure 1).

As part of the requirements for project approval, Baffinland established a Marine Environment Working Group (MEWG) of which Fisheries and Oceans Canada is a member (NIRB Project Certificate No. 005). Baffinland works with the MEWG to determine appropriate early warning indicators that will ensure rapid identification of negative impacts along the southern and northern shipping routes. To support development of an early warning indicator related to stress, the establishment of a baseline is required from which to compare against for detecting change. How early it can be detected depends on the sensitivity of the indicator relative to established threshold levels. The objective of this study is to measure cortisol levels as an indicator of stress for narwhal (*Monodon monoceros*) in Eclipse Sound across three treatments to evaluate potential effects of vessel traffic on narwhal. Here we compare narwhal samples from pre-project related vessel traffic (2000-2006) and during-project related vessel traffic (2013-2019) to determine if cortisol levels relate to increased vessel traffic in Eclipse Sound, a key narwhal summering area (Figure 1). We also compare pre- and during project related vessel traffic samples to samples from an entrapment event, which is known as high-stress and results in increased cortisol levels (Trana et al. 2016). An understanding of baseline cortisol levels and current impacts on the Eclipse Sound stock is needed to make predictions about how an increase in vessel traffic may impact narwhals in the area, and ultimately the subsistence fishery.

Methods

Sample collection and analysis

Blubber from harvested narwhal was collected by Pond Inlet hunters, hunting in Eclipse Sound, in collaboration with the Mittimatalik Hunters and Trappers Organization in Pond Inlet, Nunavut from 2000-2017 and by J. Simonee in 2019. Samples were also collected during a local harvest of narwhal from an ice entrapment in Eclipse Sound in December 2015 (for details on the entrapment event see Watt et al. 2019). Sub-dermal blubber was used for all analyses as it is known to reflect an integrated measure of stress in beluga whales (*Delphinapterus leucas*;

Loseto et al. 2018). All samples were stored frozen at -20 °C prior to analysis, and then all samples were analysed at the same laboratory within a five month time period.

Blubber (~200 mg) was transferred into a 13 × 100 culture test tube to which 5 mL of methanol and 100 µL of cortisol-d4 IS solution were added. After standing for 24 hours, the extract was concentrated. The resulting syrup was re-suspended in 2 mL 5% MeOH solution, which was filtered through a 0.2 µm syringe filter before solid phase extraction (SPE). Each SPE cartridge (Agilent Bond Elut-C18 OH) was conditioned with 3 mL 100% methanol and followed by 3 mL water at 1 mL/min. After the sample solution was loaded onto the SPE cartridge at 1 mL/min, 3 mL of 10% methanol aqueous solution was used to wash the cartridge at 1 mL/min and then 3 mL of air was flushed at 1 mL/min to dry the cartridge completely. The cartridge was then eluted with 2 mL 60% MeOH aqueous solution at 1 mL/min. The collected solution was concentrated by use of N_2 sample concentrator at 40 °C. The residue was reconstituted in 200 µL of 50% MeOH aqueous solution. After centrifuging at 14,000 ×g for 10 min, 180 µL of supernatant was injected for liquid chromatography-mass spectrometry (LC-MS) analysis.

Cortisol was purchased from Steroids Inc (Newport, RI). Deuterium labeled internal standard (IS) cortisol-d4 was obtained from CDN Isotopes Inc (Pointe-Claire, Quebec, Canada). HPLC grade methanol was purchased from Fisher Scientific (Edmonton, AB, Canada). MilliQ-H_2O was provided by Barnstead E-Pure system. Methanol aqueous solutions (5%, 10% and 60%) were prepared in the lab. Equipment used included Techne N_2 sample concentrator (Cole-Parmer, UK), Acrodisc syringe filter with Supor membrane (PALL, Corporation), 3mL Syringe (VWR, 309893), SPE cartridge (Agilent Bond Elut-C18 OH, 100MG 1mL PN: 12102020), Agilent

ZORBAX Eclipse plus C18 column (100 x 2.1 mm, 1.8 µm particle size), Agilent 1200 binary high pressure liquid chromatography and AB SCIEX QTRAP® 5500 tandem mass spectrometer.

Flow from the LC column was introduced into AB SCIEX QTRAP® 5500 equipped with an electrospray ionization (ESI) source. The mobile phase A was H_2O/ACN (95/5, v/v, 2mM NaF) and the mobile phase B was 100% ACN. The 12 min gradient was 20-40% B (0-5 min), 40% - 100% B (5-6 min), 100% B (6-8 min), 100-20% B (8-9 min), held at 20% B for 1 min. The flow rate was 220 µL/min and the injection volume was 10 µL. The electrospray voltage is 5000 v, with a temperature of 650 ℃. Curtain gas, GS1 and GS2 are 45 psi, 45 psi and 30 psi. Cortisol and the IS were detected by positive MRM (transitions 363/121, and 363/327 for cortisol and 367/121 and 367/331 for cortisol-d4, DP = 100, EP = 3, CE = 32 and CXP = 15). Calibrant solutions were prepared by serial dilution of stock solution (0.1, 0.2, 1.0, 2.0, 10, 20, 100, 200 ng/mL). Calibrations were run before and after each batch of samples and data from the standards were used to evaluate instrument performance. The calibrators were stable for at least 1 year at -20 °C. There is no matched matrix standard reference material or matrix to measure recoveries, therefore, the lowest quantifiable concentration of the standards was used as the limit of quantification. The lower limit of quantitation is 0.1 ng/mL. Standard deviations of all calibrators run in duplicate were within 3% of one another. Twenty-four blubber samples were run in duplicate and cortisol levels were within 19% of each other.

Data analysis

Cortisol levels were log-transformed to improve normality prior to data analyses. Even after log-transformation there were unequal variances between pre-project related vessel traffic (samples from 2000-2006), during project related vessel traffic (samples from 2013-2019), and entrapment blubber samples (Levene's test: $F_{2,90} = 14.31$, $p < 0.0001$). As a result, a Kruskal-Wallis test was used to compare cortisol levels across the three treatments. The Dunn post-hoc test was used to determine where significant differences occurred. Age and length of the animal was not included in this model since age of the animals was unknown, and length was often not recorded.

Results

Cortisol levels pre-project related vessel traffic ranged from 0.01 – 4.21 ng/g, with a mean of 0.81 ng/g ± 0.45 (± SE). During project related vessel traffic cortisol levels varied from 0.4 – 12.2 ng/g with a mean of 1.81 ± 0.48 ng/g (± SE). Samples from the entrapment event varied in cortisol levels from 4.13 – 23.27 ng/g, with a mean of 10.52 ± 0.59 ng/g (± SE). There was a significant difference among blubber cortisol levels in pre-project related vessel traffic (n = 38), during project related vessel traffic (n = 33), and entrapment (n = 22) samples ($X^2_2 = 55.13$, $p < 0.0001$). Dunn post-hoc tests showed a significant difference between all treatments, with blubber samples taken pre-project related vessel traffic having significantly lower cortisol levels (0.81 ± 0.45 ng/g [±SE]) than those collected during project related vessel traffic (1.81 ± 0.48 ng/g [±SE]) (Z = 2.74, p < 0.05) or entrapment samples (10.52 ± 0.59 ng/g [±SE]) (Z = 7.41, p < 0.001). Entrapment samples had significantly higher cortisol levels than samples collected during project related vessel traffic (Z = 4.84, p < 0.001) (Figure 2). Variability in pre-project related vessel traffic was greater (SD = 0.70) than during project related vessel traffic (SD = 0.30) (Figure 2).

Discussion

There was an increase in cortisol levels from the pre- to during project related vessel traffic in narwhal blubber sampled in Eclipse Sound, NU. Samples from entrapped narwhals had significantly higher cortisol levels than samples from pre- and during project related vessel traffic, indicative of the high stress experienced during these events as a result of limited access to the surface for breathing and inhibited ability to forage (Siegstad and Heide-Jørgensen 1994). Vessel traffic, although not as stressful as an ice entrapment event, is ongoing long-term and could result in chronic stress over time. Variability in cortisol levels among individuals was reduced during project related vessel traffic, which may suggest that stress induced by vessel traffic is masking some of the individual differences in cortisol levels that may have been attributed to other environmental stressors pre-project related vessel traffic. Vessel traffic will continue to increase in the Arctic into the 21st century (Stephenson et al. 2013), and this may lead to chronic stress and reduced reproductive success in narwhals in the region (Sheriff et al. 2010; Dantzer et al. 2014).

The defined time periods for pre- and during- project related vessel traffic were based on sample availability and information on vessel traffic from Baffinland. However, there was also a marked increase in vessel traffic overall in the Arctic after the former ice-minimum in 2007 (Pizzolato et al. 2014), which also corresponds to the time periods assessed as pre- and during project related vessel traffic in the present study. In addition, the average distance travelled by vessels in the Tallurutiup Imanga National Marine Conservation Area in the Canadian Arctic doubled between the two time periods of this study (Figure 3). Kochanowicz et al. (2020) found that for the period 2000-2006, the average distance vessels travelled was 58,000 km while the average distance was 117,000 km for the period 2014 to 2018 (the study did not include data from 2019, Figure 3). Dawson et al. (2018) also found that vessel traffic was stable with little growth from 1990 to 2000, suggesting samples from this time period could be combined for analysis and act as a baseline for cortisol levels.

Vessel traffic is considered a major stressor for marine mammals and could lead to reduced feeding, communication, and navigation ability that can ultimately lead to reduced fitness (Wright et al. 2007). Vessel noise produced at similar frequencies made by whale vocalizations can mask communication and disrupt social interactions (Pine et al. 2020). Relatively little is known about narwhal behavioural responses to vessel traffic, but Finley et al. (1990) found that narwhal avoid icebreakers, and beluga whales are known to avoid boat noise and decrease vocalizations (Halliday et al. 2019); our study did not consider behavioural impacts in response to vessel traffic, and this should be evaluated in the future. Older archived blubber samples that have visibly degraded are known to have lower cortisol levels (Trana 2014) and as a result we limited our sample analysis to 2000 where no visible degradation occurred to remove the bias of sample degradation. As a result, we recommend this pre-vessel traffic time period be used as a baseline of cortisol level in future studies and that there is continued monitoring of cortisol levels in narwhal stocks that are susceptible to interactions with high vessel traffic.

Cortisol levels vary among individuals and can be influenced by many biological factors including the age, size, sex, and reproductive status of an animal (Suzuki et al. 2013; Azevedo et al. 2019). Unfortunately we were unable to evaluate these biological factors for narwhal cortisol levels with the present data. However, Loseto et al. (2018) found that in beluga whales, outer blubber cortisol levels were not significantly effected by these biological factors and recommended that this is an exemplar tissue for monitoring. Although there is a correlation between cortisol levels and project related vessel traffic, we are unable to determine the cause and effect of higher cortisol levels during project-related vessel traffic; monitoring of cortisol levels in outer blubber is recommended as vessel traffic continues to increase.

There have been few studies of cortisol level in narwhal, however, some studies have been conducted on their closest relative, the beluga whale. In beluga whales, cortisol levels of entrapped whales averaged 1.76 ng/g (Trana et al. 2016), whereas in the narwhals sampled here, mean cortisol level was 10.52 ng/g. Trana et al.'s (2016) study used hormone assays to measure cortisol, while we used LC-MS; although LC-MS is more sensitive than assays, there is typically no significant difference between results from the two methods (Bowerbank et al. 2019). This suggest stress was significantly higher in entrapped narwhals compared to belugas. This could result from different timing of entrapment events and the length of time animals were entrapped prior to being sampled. Healthy, harvested samples from beluga whales from the western Canadian Arctic had similar cortisol levels as low stress narwhal samples from 2000-2006 in this study, ~0.8 ng/g in both narwhals and beluga whales (Loseto et al. 2018). Blubber from narwhals sampled during the vessel traffic period in the Eclipse Sound region was over 100% higher in cortisol than samples from the pre-project related vessel traffic period, and higher than beluga cortisol levels during an entrapment event (Trana et al. 2016).

Whether this increase in chronic stress has already occurred or will impact reproductive success of narwhals from the Eclipse Sound stock in the future is unknown. Therefore, monitoring of reproductive rates, narwhal body condition, and population abundance will be important to assess the long-term impacts of increased stress levels (Booth et al 2020). It is difficult to determine what the root cause of an increase in stress may be. Changing ice conditions (Laidre and Heide-Jorgensen 2005), changes to Arctic food webs (Post et al. 2013), an increase in killer whales in the Canadian Arctic (Higdon and Ferguson 2009), and/or increased vessel traffic (Eguíluz et al. 2016), could all contribute to increasing stress levels for narwhals. However, the cumulative impact of all these changes may have a larger impact on narwhal populations than any one factor alone. Cortisol levels should be used as an early indicator of stress and continued monitoring of cortisol levels in narwhals is warranted. Studies linking stress and reproductive success would assist with future population projections.

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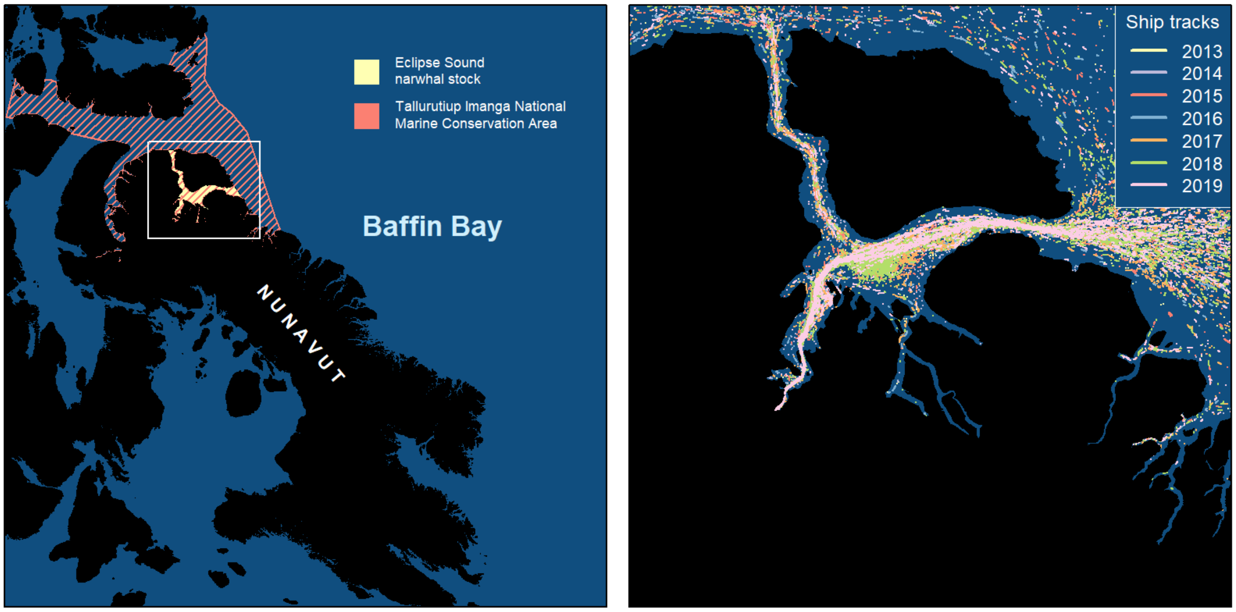

Figure 1. Distribution of the Eclipse Sound narwhal stock (left panel) and vessel locations for the area from 2013-2019 (right panel). The Tallurutiup Imanga National Marine Conservation Area is highlighted for reference.

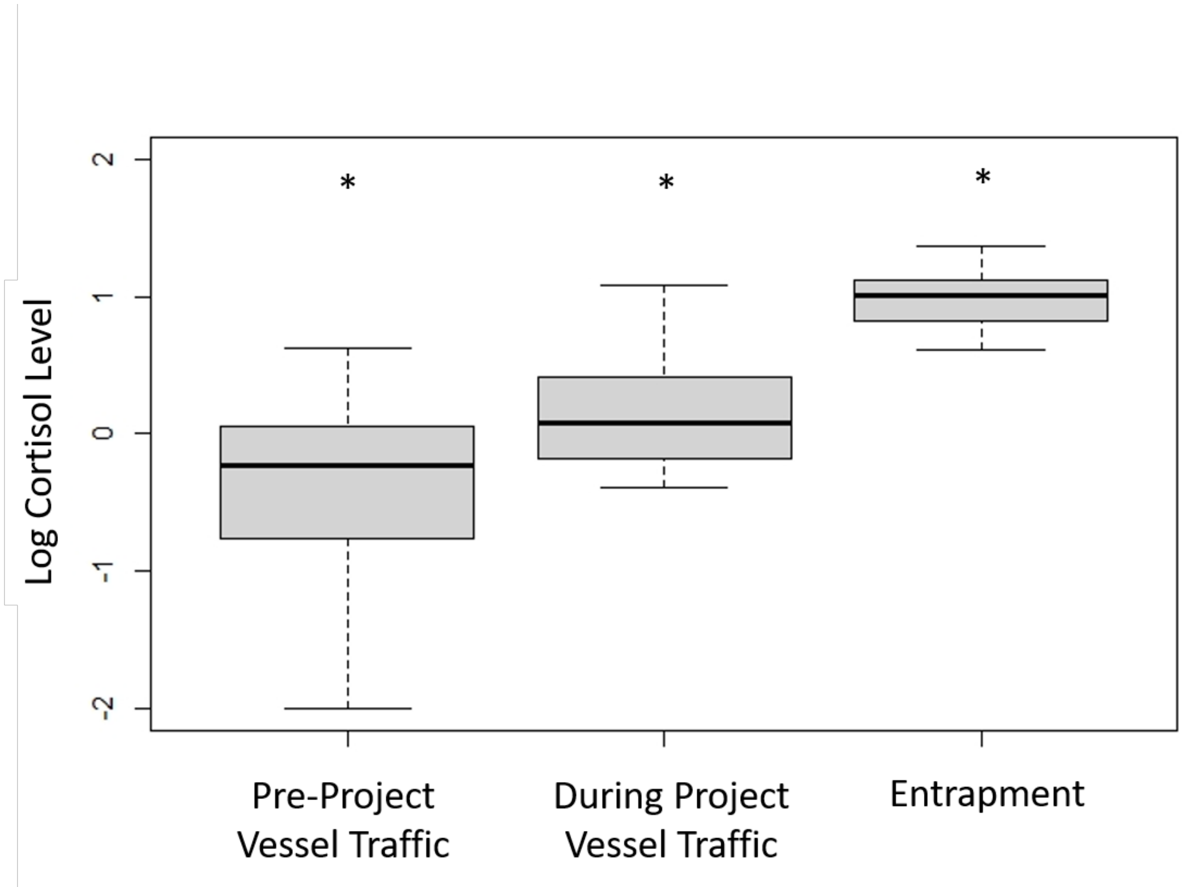

Figure 2. Boxplot of log-transformed cortisol level for three treatments, pre-project related vessel traffic (2000-2006), during project related vessel traffic (2013-2019), and an entrapment event (2015). Asterisks indicate significant differences among all three treatments.

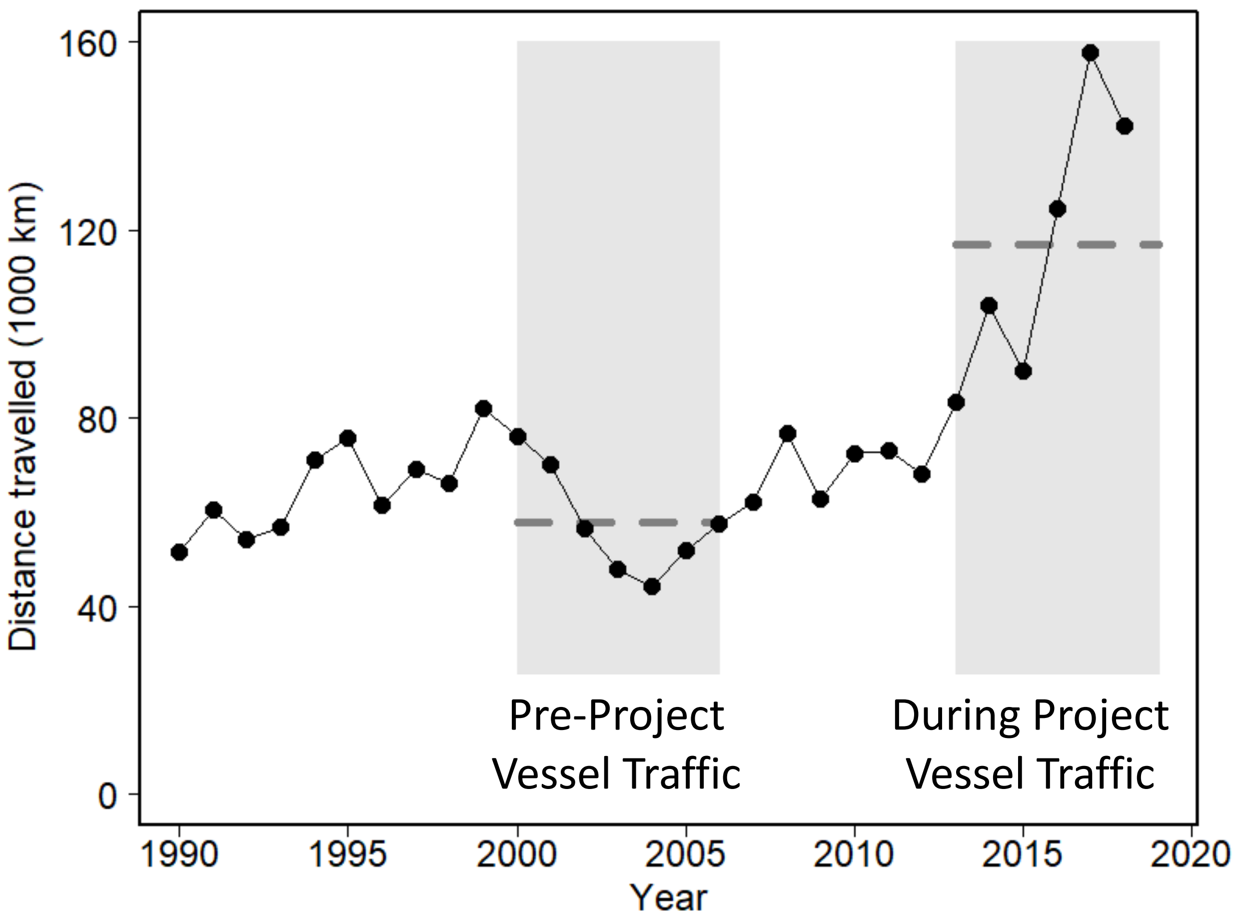

Figure 3. Total distance travelled by vessels transiting the Tallurutiup Imanga National Marine Conservation Area from 1990-2018. Data from Kochanowicz et al. 2020.