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In-depth analysis of live-stranded long-finned pilot whale samples: Stable isotope and genetic investigation of a fatal long-finned pilot whale mass stranding event (Isle of Lewis, 2023)

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Summary

The mass live-stranding of 55 long-finned pilot whales (*Globicephala melas*) on 16 July 2023, at Tolsta Beach, Isle of Lewis (Outer Hebrides, Scotland), represents a rare and scientifically valuable opportunity to investigate the health, ecology, and potential causes of stranding in this data-deficient species. This event, the largest recorded fatal mass stranding event (MSE) in Scotland since 1927, provided access to tissue samples from relatively healthy animals. This offers a unique contrast to samples from many dead-stranded individuals, which are often biased by unknown factors contributing to their stranding. The comprehensive necropsies (n = 23) and extensive tissue sampling (n = 54 individuals, including an additional three foetuses) conducted shortly after the stranding enabled the collection of high-quality biological material and metadata, including age class, sex, reproductive status, and cause of death. These samples, combined with multidisciplinary analyses (genetic, isotopic, fatty acid, metabolomic, pathological, and microbiological), provide an unprecedented opportunity to address critical knowledge gaps in the ecology and conservation of long-finned pilot whales (LFPW) in UK waters.

The project addresses four key research questions:

1. Were all LFPW feeding on the same prey in the same location prior to the MSE?
2. Were multiple pods involved in the MSE?
3. Did their diet shift in the weeks or months preceding the MSE?
4. Were the stranded animals in good nutritional condition prior to the event?

This project identified that the animals involved in the MSE were in good nutritional condition and did not appear to show signs of a sudden dietary shift that could equate to a change in foraging depth/habitat type. The Isle of Lewis MSE contained multiple pod units (families) of LFPW, that were unrelated to each other. The presence of multiple unrelated pod units in July, which is within LFPW breeding season, indicates that, prior to the MSE, this large assembly of LFPW were likely in a multigroup association for breeding purposes. This conclusion is also supported by genetic evidence from the subsequent 2024 Orkney MSE, which was also analysed genetically as part of this work, results can be found in Appendix 2. The combined isotope and genetic analysis for the Isle of Lewis MSE revealed that pod units showed distinct spatial use of resources (via skin $\delta^{13}C$ and $\delta^{34}S$), furthering the evidence for multigroup association prior to this MSE. The presence of juvenile animals without mothers or closely related pod members indicates that this mass stranding event (large as it was) may not have included all animals from all pods that were present in the immediate area that day. Annual multigroup aggregation for breeding purposes from June to August has implications for marine mammal management in northwest Scotland. The results of this project enhance our understanding of LFPW ecology, provide a framework for interpreting future stranding events, and have the potential to inform the conservation of deep-diving cetaceans in the face of cumulative anthropogenic and climatic pressures.

Contents

Acronyms

List of acronyms used throughout this report.

Acronym	Definition
MSE	Mass Stranding Event
LFPW	Long-finned Pilot Whale
PUFA	Polyunsaturated Fatty Acids
SMASS	Scottish Marine Animal Stranding Scheme
MPA	Marine Protected Area
NEIF	National Environmental Isotope Facility
VPDB	Vienna Pee Dee Belemnite
AIR	Air
VCDT	Vienna Canyon Diablo Troilite
ASE	Accelerated Solvent Extractor
TLE	Total Lipid Extract
FAMEs	Fatty Acid Methyl Esters
GC	Gas Chromatograph
CDS	Chromatography Data System
SIBER	Stable Isotope Bayesian Ellipses
SEA	Standard Ellipse Area
MUFA	Monounsaturated Fatty Acids
PUFA	Polyunsaturated Fatty Acids
DNA	Deoxyribonucleic Acid
HWE	Hardy-Weinberg Equilibrium
NeA	Northeastern Atlantic
Ho	observed heterozygosity
He	expected heterozygosity
Na	Number of different alleles
AMOVA	Analysis of Molecular Variance
PCA	Principle Components Analysis
LOD	Log Odd Ratios

1. Introduction

Long-finned pilot whale (LFPW) are highly social cetaceans with matrilineal leadership and intergenerational support, primarily provided by female pod members (Boran & Heimlich 2019; Zwamborn, Walmsley & Whitehead 2023). Behavioural studies indicate that pods typically consist of three to 14 closely bonded individuals (pod unit), likely comprising immediate kin or long-term social companions (Augusto, Frasier & Whitehead 2017b; de Stephanis *et al*. 2008; Ottensmeyer & Whitehead 2003). These pod units form pod complexes, which are larger, stable social groups composed of multiple related females and their offspring. Within these pod complexes, alloparental care has been observed from both males and females (Augusto, Frasier & Whitehead 2017a).

The species predominantly inhabit deep waters (> 500 m) and forage for deep-sea squid (Ottensmeyer & Whitehead 2003; Santos *et al*. 2014). Their strong social bonds, synchronous diving, and milling/pod aggregation behaviours in response to stressors make them highly susceptible to mass stranding events (MSEs; Ball *et al.* 2021; Visser *et al.* 2016). MSEs are often triggered by:

- predator interactions (e.g. response to killer whale vocalizations),
- disorientation from noise pollution (e.g. mid-frequency naval sonar),
- distress or illness in a pod leader (Visser *et al.* 2016), and
- disruption to social bonds in large gatherings (Oremus *et al.* 2013).

The primary objective of this project is to use multidisciplinary approaches to investigate the 16 July 2023, mass stranding of 55 LFPW on Tolsta, Isle of Lewis. This MSE investigation seeks to assist in the evaluation of the potential cause(s) of the MSE, distinguishing between natural (e.g. disease, predation interaction, navigational errors) and anthropogenic (e.g. naval sonar, pollution, fisheries interactions) drivers. By integrating stable isotope, fatty acid and genetic data with pathological and microbiological findings, the study will assess whether the stranded animals exhibited signs of acute or chronic stressors that could have contributed to the event. This information will be essential for informing marine management strategies and mitigating future stranding risks.

1.1. Long-finned pilot whale (LFPW) mass stranding events (MSE)

To date, there have been eight LFPW MSE that have occurred in Scotland since 2011:

- 22 July 2011 Kyle of Durness, 16 fatalities
- 2 September 2012 Pittenweem, 23 fatalities
- 24 April 2013 Inverness, 2 fatalities
- 2 June 2015 Staffin Island, 9 fatalities
- 6 December 2018 Culross, 4 fatalities
- 12 June 2020 Lochboisdale, 13 fatalities
- **16 July 2023 Tolsta, 54 fatalities**
- 11 July 2024 Sanday, 77 fatalities

Prior to 2023, the largest long-finned pilot whale MSE in Scotland was Pittenweem (Fife) in 2012, with 23 fatalities. At the time, the 2023 event at Tolsta Beach on the Isle of Lewis was the largest recorded fatal cetacean mass stranding in Scotland since 1927 (Tomlin 1957). The 2023 MSE (Figure 1) provided access to tissue samples from relatively healthy animals, offering a unique contrast to samples from dead-stranded single individuals, which are often biased by unknown factors contributing to their stranding. Multidisciplinary analyses (genetic, isotopic, fatty acid, metabolomic, pathological, and microbiological) provide an unprecedented opportunity to address critical knowledge gaps in the ecology and conservation of LFPW in UK waters.

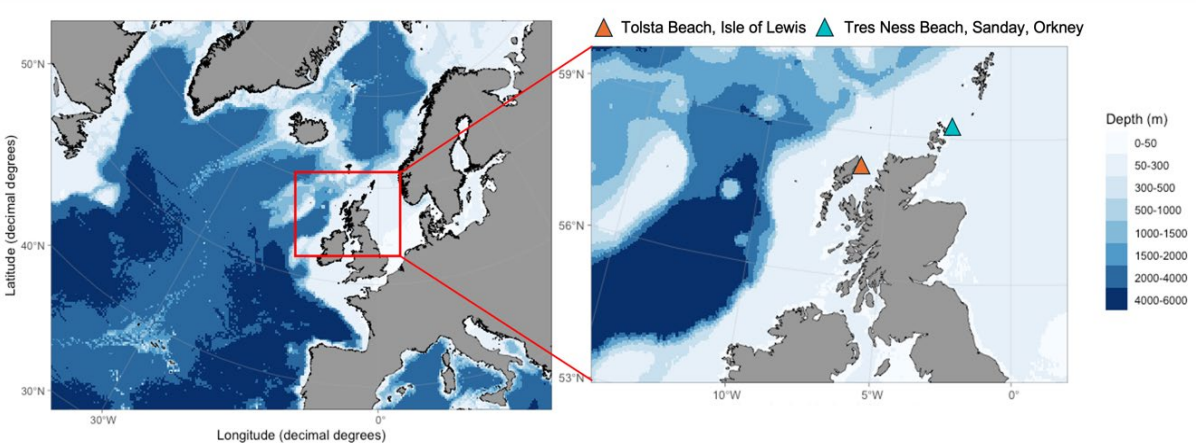

Figure 1. Map of the North Atlantic showing the locations of the two most recent fatal mass stranding events that took place in northern Scotland in 2023 and 2024. Map made using ggOceanMaps in R (Vihtakari, 2024).

1.1.1. Tolsta Beach, Isle of Lewis (2023) MSE

On 16 July 2023, a MSE involving 55 LFPW occurred at Tolsta Beach, Isle of Lewis. Despite large-scale community rescue efforts, only one individual was successfully refloated. Following eight hours of refloating attempts, deteriorating weather conditions and dangerous surf, the remaining surviving whales (*n* = 12) were euthanised by a local veterinarian on welfare grounds. Necropsies (*n* = 23) and tissue sampling (*n* = 53 + 3 foetuses) were conducted shortly after the euthanasia (Table 1). A multidisciplinary investigation, led by the Scottish Marine Animal Stranding Scheme (SMASS) in collaboration with multiple UK institutions, is ongoing to assess the health and behaviour of the stranded whales and determine whether natural or anthropogenic factors contributed to the event. The Isle of Lewis 2023 MSE occurred on a remote soft-sand beach (Figure 2), limiting rescue opportunities and self-rescue potential on tidal cycles and occurred at the top of the tide cycle.

Figure 2. Initial discovery of 55 stranded LFPW on Tolsta Beach, Isle of Lewis (Photo: M. ten Doeschate, 16 July 2023).

Table 1. Summary of individuals involved in the Isle of Lewis 2023 MSE, and the samples collected.

Globicephala melas	**Isle of Lewis MSE**
Date	16 July 2024
Total Animals	55
Animals Alive on Discovery	12
Animals Euthanised	12
Animals Refloated	1
Deceased Male	17 (6 adults, 9 juveniles, 2 foetus) (alive – 1 juvenile refloated)
Deceased Female	40 (30 adults, 8 juveniles, 1 neonate, 1 foetus)
Necropsies	23 total (18 females, 5 males)
% Necropsied	42
% Necropsied or Sampled	98
Number of Samples Collected	1720

1.2. Stable Isotope and Fatty Acid Niche Modelling

In this project, we use molecular data (stable isotopes and fatty acids) to understand what, where, and how well long-finned pilot whales were eating before they were involved in a fatal mass stranding event. This information helps us understand why the animals stranded and if food or feeding success played a role. Stable isotope and fatty acid analyses are powerful remote ecological monitoring tools that provide insights into the foraging niche, diet composition, and habitat use of marine mammals. These methods can reveal trophic level within the food web, habitat use (e.g. coastal, pelagic, benthic), preferred prey species and their proportional dietary contribution, seasonal and ontogenetic (age-related) dietary shifts, and nutritional condition and foraging success.

This analysis also aims to establish stable isotope and fatty acid analysis as a viable tool for long-term, remote monitoring of data-deficient cetacean species in offshore and deep-sea ecosystems. By generating baseline ecological data for LFPW in Scottish waters, this study can contribute to the evidence base for marine protected area (MPA) management, fisheries policies, and conservation strategies under the UK Marine Strategy, OSPAR, and the Habitat Regulations. Additionally, the methods developed in this project may be applicable to other deep-diving and elusive marine mammals, thereby enhancing our capacity to monitor and protect these species in the face of cumulative anthropogenic and climatic pressures.

1.2.1. Stable Isotope Analysis

The principle "You are what and where you eat" underlies stable isotope analysis. Carbon, nitrogen, and sulphur isotopic signatures integrate information about diet and habitat use into animal tissues at different rates (DeNiro & Epstein 1978, 1981; Connolly *et al.* 2004). Tissue-specific isotope turnover rates provide dietary records over different time scales (Teixeira *et al.* 2022):

- Liver: days to weeks
- Skin: weeks to months
- Muscle: months
- Bone: years

1.2.2. Fatty Acid Analysis

Fatty acids (FA) are essential to all life. Fatty acids (stored in neutral lipids within the body) provide metabolic energy when glucose supplies run low, form cellular membranes, and act as precursors for crucial hormonal and signalling compounds (reviewed in Bond *et al.* 2016). A similar “you are what you eat” concept applies to the fat molecules found in marine mammal blubber energy reserves. These fat molecules are composed of smaller fatty acid “building-blocks”. In mammals, these fatty acid building-blocks come from two different sources:

- Internal biosynthesis (made within the body).
- Directly incorporated from food.

The specific types of fat molecules found in blubber (particularly polyunsaturated fatty acids or PUFA), can be used as indicators (or “biomarkers”) of what kind of prey species the whale has eaten. This can tell us about the “what and where” of a whale’s feeding behaviour (Saito *et al.* 2002; Budge *et al.* 2006). PUFA building blocks that come from diet are more likely to

be stored in the inner (closest to the muscle) part of the whale's blubber layer (Strandberg *et al.* 2008). The proportion of PUFA from diet relative to internally synthesised FA can tell us how successfully the animal has been feeding ("nutritional condition") (Plint, unpublished PhD data).

Overall, stable isotope and fatty acid analysis have been successfully applied in marine mammal ecology to determine:

- What and where animals hunt (Hooker *et al.* 2001; Giménez *et al.* 2017; Borrell *et al.* 2021).
- Age-related changes in diet (Knoff, Hohn & Macko, 2008).
- How long mothers nurse their young (Feyrer *et al.* 2020).
- How different marine mammal species share the same space (Fernández *et al.* 2011; MacKenzie *et al.* 2022).
- What kind of marine pollution they are exposed to (Pinzone *et al.* 2019; Remili *et al.* 2021).
- Individual animal hunting strategies (Krahn *et al.* 2007; Jory *et al.* 2021; Remili *et al.* 2023).

1.3. Genetic Analysis

Genetic analyses can be particularly powerful to make inferences about connectivity between populations (i.e. population structure) and relatedness in marine animals due to the difficulty of physically tracking their movements and behaviours (Banguera-Hinestroza *et al.* 2014; Martien *et al.* 2017). The ability to sample many individuals from a Mass Stranding Event (MSE) provides the opportunity to gather detailed information on morphological and physiological features such as sex, length, reproductive state and diet.

1.3.1. Population Structure

Genetic inferences about population structure assess the relative variation within populations (diversity) and the distinctiveness between populations (differentiation). Such measures can be used to make inferences about the degree of interbreeding between populations (gene flow), to deduce, for example, the spatial scales over which connectivity occurs (Durante *et al.* 2022; Kersten *et al.* 2021). Identifying the degree of connectivity between populations within a species range is essential for developing conservation strategies and management plans (Quérouil *et al.* 2007; Attard *et al.* 2018; Kersten *et al.* 2021). Isolated populations could be more at risk of losing genetic variation through inbreeding due to a small number of breeding partners, which could result in a reduced ability to adapt to changing environmental conditions (Hohenlohe, Funk & Rajora 2021).

For marine mammals that typically are not separated by physical barriers to movement, genetic connectivity can be driven by a range of factors, including environmental (Vargas-Fonseca *et al.* 2021), habitat requirement and size (Louis *et al.* 2014), prey specialisation (Onoufriou *et al.* 2022) and social behaviour (Van Cise *et al.* 2017; Martien *et al.* 2019).

1.3.2. Relatedness

MSEs provide a unique insight into the group dynamics of such events, especially as many of these events are seen in species thought to form strong social bonds within a matrilineal (mother and her female line descendants) social structure (Norris & Schilt 1988; Oremus *et al.* 2013). Social bonds and learning especially between mother-calf pairs in these matrilineal pods, form a vital part of many cetacean group dynamics (Alves *et al.* 2013; Whitehead 2017; Sarano *et al.* 2021).

The matrilineal social structure of cetaceans that mass strand, points to kinship as a potential underlying force during an MSE, where individuals exert effort to remain with close relatives. The theory within the literature states that MSE are likely to be made-up of either individuals that are part of an “extended matriline” (all individuals are related to one maternal ancestor) OR “multiple matrilines” (large groups represent temporary associations of smaller social units characterised by different maternal lines) (Amos, Schlotterer & Tautz 1993; de Stephanis *et al.* 2008; Oremus *et al.* 2013).

Establishing relatedness of individuals within MSEs can thus be highly informative about the strength of such social bonds and the relationships between individuals associating prior to the stranding event (Oremus *et al.* 2013). Within the MSE, regardless of which theory the individuals exhibit, the “kinship cohesion” hypothesis predicts that close relatives will maintain close proximity to each other when they strand, particularly mother-juvenile pairs between which the social bond is expected to be the strongest (Oremus *et al.* 2013). Using genetics along with location data is the only way to identify mother-juvenile pairs and determine their proximity on the beach.

2. Stable Isotopes and Fatty Acids (Objective 1)

The first objective of this project is to employ stable isotope and fatty acid analyses to reconstruct the ecological niche, diet, and habitat use of LFPW involved in the 2023 Isle of Lewis MSE. This study aims to provide insights into the trophic level, foraging behaviour, and nutritional condition of these animals in the weeks to months preceding the MSE. The resulting data will be compared with samples from single stranded individuals affected by chronic illness or starvation to establish contrasting values for healthy relative to compromised individuals, enabling a more comprehensive understanding of the health status of the stranded group.

2.1. Aims

We assessed individual and collective foraging niche and nutritional status of long-finned pilot whales involved in the 2023 Isle of Lewis MSE using muscle, skin, and liver $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ and blubber fatty acid (FA) composition to elucidate aspects of their social connectivity, behaviour, and foraging success leading up to the stranding event. Specifically, we aimed to identify both a) the foraging niche of the MSE animals and b) the nutritional condition of the animals prior to stranding to address the following questions:

Q1. Were all 54 LFPW feeding on the same prey, in the same place before the MSE?

H1. The 54 animals likely represent multiple groups, with different diet and movement patterns prior to aggregating and stranding in response to stressor(s). We expect to see calves/young juveniles retain a full or partial weaning signal.

Q2. Were multiple pods involved in the MSE?

H2. Paired isotopic and genetic data are expected to reveal multiple established pod units that travelled/foraged separately prior to the MSE.

Q3. Has their diet changed recently (i.e. weeks to days before the MSE)?

H3. Short-term LFPW diet changed suddenly with a shift from deep-water shelf-edge to shallower shelf-seas leading up to the MSE. Preceding this, we expect a gradual Spring-Summer seasonal shift in isotopic signatures across the tissue timeseries.

Q4. Have the MSE LFPW been feeding well recently?

H4. Tissue isotope and blubber FA composition will show some interindividual variation in diet and nutritional condition based on individual animal age and health. However, consistent with blubber depth measurements, we do not expect the blubber fatty acid profile of any MSE animals to show signs of chronic poor nutritional condition/starvation.

2.2. Methods

2.2.1. Sampling methodology

Full-depth blubber profile (n = 50), muscle (n = 46), skin (n = 53), and liver (n = 46) subsamples were collected from animals involved in the 2023 Isle of Lewis MSE. In some cases, not every tissue sample was available for every animal. Additional blubber profiles from poor body condition single-stranded animals (n = 4; 2017–2024) were subsampled for nutritional condition comparison. Samples were held in dedicated -20°C SMASS archives until analysis. The scalpel was cleaned with a solvent prior to each sample to prevent lipid

contamination from the underlying blubber layer. Inner and outer blubber layers were separated and analysed separately.

2.2.1.1. Sample preparation for CNS isotope analysis

Pilot whale muscle, skin, and liver samples were desiccated at 60°C, powdered, and weighed into pressed tin capsules (0.8 mg). A subset of samples (*n* = 10/tissue type) was separated into two aliquots to determine tissue lipid content and create a mathematical correction factor for the remaining samples to account for the impact of lipid $\delta^{13}C$ on tissue protein $\delta^{13}C$ (McConnaughey & McRoy 1979; Post *et al.* 2007). Following a modified Bligh and Dyer (1959) method, one aliquot underwent a lipid extraction process (three rinses of 30 minutes each in 2:1 chloroform:methanol) prior to drying at 60°C. The paired aliquots underwent no lipid extraction to avoid any deleterious effect of chemical extraction on collagen amino acids and their resultant $\delta^{15}N$ value (Smith *et al.* 2020).

2.2.2. Stable isotope analysis

The carbon, nitrogen, and sulphur stable isotope compositions ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) of pilot whale tissues were measured using an Elementar Pyrocube elemental analyser (EA), coupled to a ThermoScientific™ Delta Plus XP isotope mass spectrometer *via* a ConFlo IV system, using helium as the carrier gas at the National Environmental Isotope Facility (NEIF) isotope ecology laboratory. All isotope results are reported in δ-notation in per mil (‰) relative to international standards calibrated to VPDB, AIR, and VCDT.

2.2.3. Blubber lipid extraction

Paired inner and outer blubber sample sets (*n* = 15) were lipid extracted following Magill *et al.* (2015) using an Accelerated Solvent Extractor (Dionex ASE 350(ASE)). Samples were placed in pre-extracted cells and lipid extracted with 5:1 dichloromethane:methanol (DCM:MeOH), set to three cycles of five minutes at 100°C, with a rinse volume of 75%. Due to technical difficulties with the ASE, the remaining inner-outer blubber sample sets (*n* = 35 MSE animals and 4 single-stranded animals) were lipid extracted using three rinses of 30 minutes each in 2:1 chloroform:methanol in an ultrasonic bath. The resultant blubber total lipid extract (TLE) was transferred to 4 mL vials, dried to a film under gentle N_2 stream, and then stored at -20°C until further analysis. TLE was resuspended in hexane at a concentration of 10 mg/mL.

2.2.4. Fatty acid methyl esterification

Blubber fatty acids were isolated for identification and quantification. The neutral lipid fraction (e.g. sterols, wax esters, triacylglycerols) from each tissue sample was subjected to acid methanolysis to cleave intact neutral lipids and transesterify them into component Fatty Acid Methyl Esters (FAMEs). Hydrolysis was achieved through lipid reaction with 250 µL of 0.5 N butanolic hydrochloric acid at 60°C for ~16 hours. The hydrolysis reaction was quenched with 250 µL of DCM-extracted ultra-clean Millipore water. Subsequent liquid-liquid extraction of the organics was performed using four rinses of 500 µL of 4:1 vol/vol hexane:DCM. Resultant FAMEs were dried under gentle N_2 stream and then resuspended in 1.5 mL of hexane.

2.2.5. Fatty acid quantification

Molecular identification of the FAMEs was performed using a ThermoScientific Trace 1300 gas chromatograph (GC) and TriPlus RSH autosampler, coupled to an ISQ LT Single quadrupole Mass Spectrometer (electron ionization mode; mass range: 50 to 600 amu), with helium as the carrier gas. Sample injection volume was 1 µL. Front inlet (Programmable

Temperature Vaporizer) temperature was 300°C. The GC was equipped with a fused silica 30 m DB-5HT column (0.25 mm x 0.10 µm). The GC column oven temperature program started at 60°C, with an oven ramp of 6°C/minute to a maximum temperature of 320°C, for a hold time of 20 minutes. The reference materials Fatty Acid Ester Mixture F8-3 and Supelco37 (pure fatty acid methyl and ethyl esters) were included at the beginning and end of every analytical session to map compound elution and retention time. Processing and integration software was ThermoScientific Chromeleon Chromatography Data System (CDS) version 7.3.1. FAMEs were identified using mass fragmentation patterns and parent ions published on The Lipid Web (www.lipidmaps.org) and NIST (National Institute of Standards & Technology) mass spectral library (https://webbook.nist.gov/chemistry/name-ser/). Fatty acids are reported as (%) relative abundance of all identified compounds.

2.2.6. Data Analysis

Data analyses were performed using R version 4.2.1 (R Core Team 2025). Long-finned pilot whale isotopic niches were determined using the SIBER package (Stable Isotope Bayesian Ellipses in R; Jackson *et al.* 2011). SIBER creates isotopic niche models of consumer groups (e.g. species, age class, sex) using Bayesian multivariate normal distributions, calculating core isotopic niche (ellipses constraining 40% of data per species - Standard Ellipse Area (SEA) corrected for small sample size [SEA_C]) and total isotopic niche (convex hull containing 100% of data per species - Total Area [TA]). Fatty acid composition by blubber layer was visualised on log10 transformed data using ggplot (geom_jitter function).

2.3. Results and Discussion

2.3.1. Long-finned pilot whale stable isotope signatures

The mean and range $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ of liver, skin, and muscle are reported in Table 2. To negate the influence of lipids on the protein carbon isotope composition, the $\delta^{13}C$ values of all three tissues were lipid-corrected using the equation following Post *et al.* (2007). As expected, residual weaning signal and trophic differences in $\delta^{15}N$ between age classes drove intraspecific variation within the group of animals (Figures 3 and 4).

This study added new data to what is known about the pelagic delphinid community in Scottish waters (Figure 5; Plint *et al.* 2023). We found that long-finned pilot whales occupy a distinct isotopic foraging niche and show core niche overlap with striped dolphins, suggesting similar deep-water shelf-edge habitat and potential overlap in prey type. Long-finned pilot whale $\delta^{13}C$ range overlaps with Risso's and white-beaked dolphin in Scottish waters, reflecting their shared deep-water foraging habitat.

In the broader context of the Northeast Atlantic, the MSE animals are isotopically highly similar to LFPW stranded in Iceland (1988–2021) and Scotland (1992–2012) (Monteiro *et al.* 2015; Samarra *et al.* 2024). They are isotopically distinct from LFPW sampled in the Bay of Biscay and the Mediterranean (Monteiro *et al.* 2015; Pinzone *et al.* 2019). As such, the MSE animals were presumably resident to the northeast Atlantic waters around Scotland. We propose that any small differences in isotopic signal between LFPW collected in Scotland between 1992–2012 and those involved in the MSE could be explained by seasonal changes in diet or environment (for example, Espinasse *et al.* 2022).

Table 2. Mean ± 1SD $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ values for long-finned pilot whale liver, skin, and muscle samples collected from the mass stranding event.

Tissue	Mean $\delta^{13}C$ (‰) VPDB	Mean $\delta^{15}N$ (‰) AIR	Mean $\delta^{34}S$ (‰) VCDT
Liver	–19.7 ± 0.5	+12.2 ± 0.5	+17.9 ± 0.3
Skin	–17.3 ± 0.9	+11.0 ± 0.7	+18.3 ± 0.3
Muscle	–17.0 ± 0.5	+10.9 ± 0.7	+17.7 ± 0.5

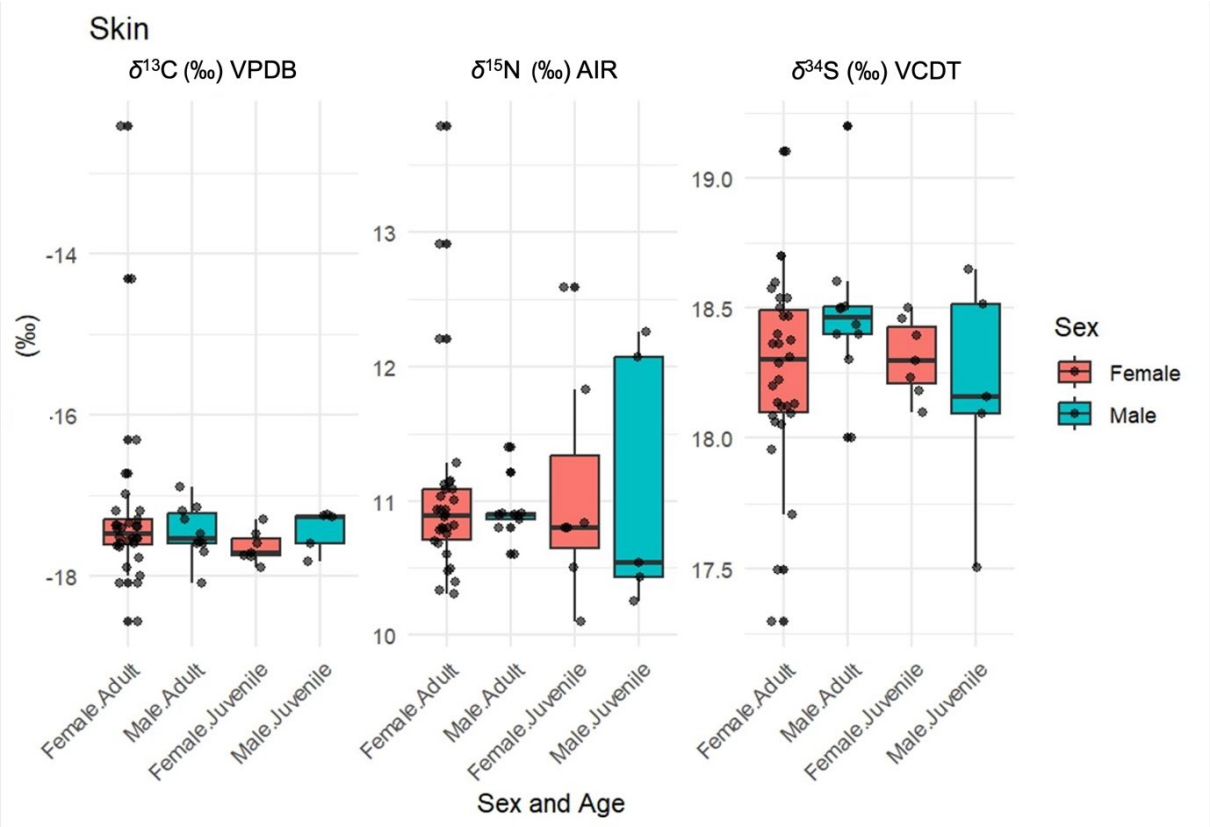

Figure 3. Carbon, nitrogen, and sulphur isotopic variation in long-finned pilot whale skin tissue based on sex and age class.

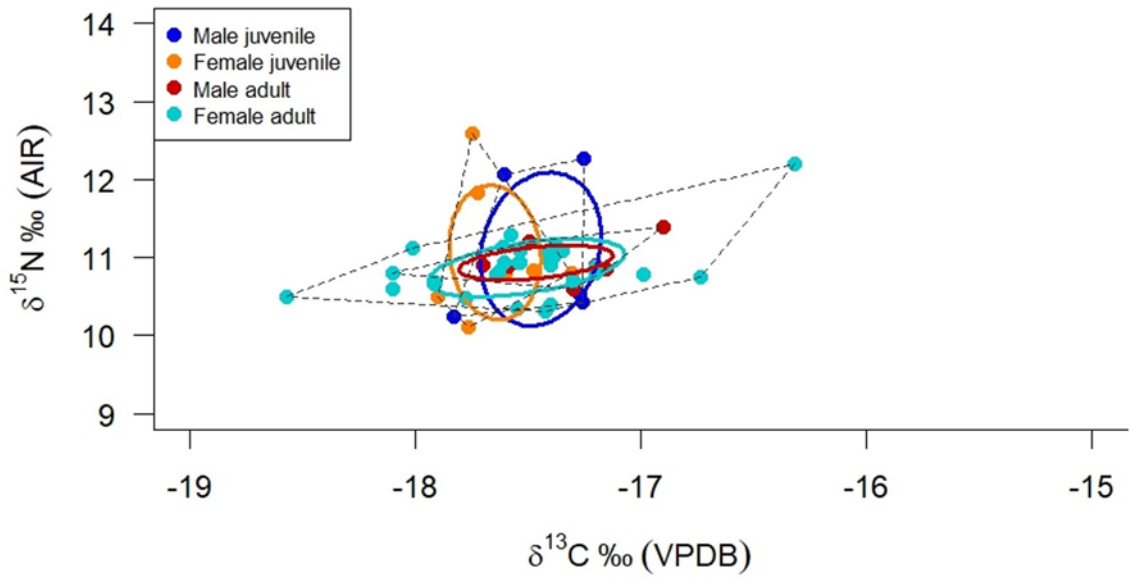

Figure 4. Core and total isotopic foraging niche differences (based on skin δ13C and δ15N) for long-finned pilot whales in Scottish waters, according to sex and age class.

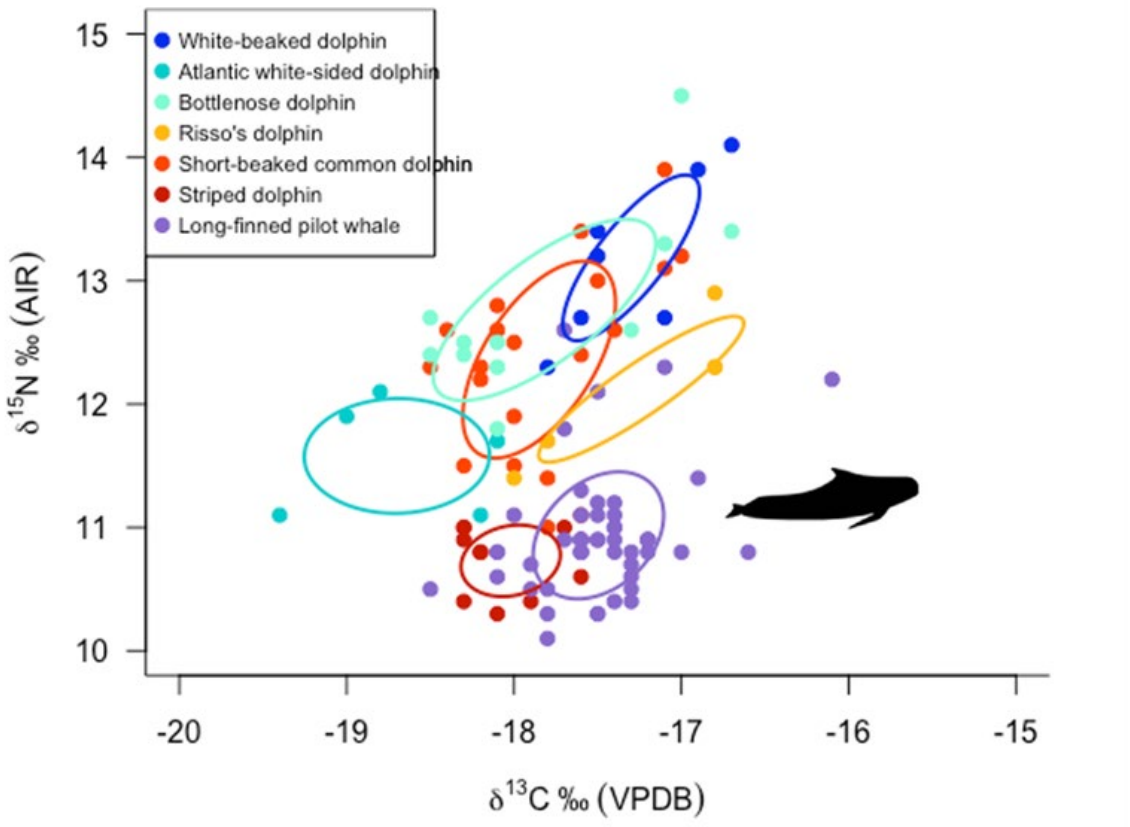

Figure 5. Core isotopic foraging niches for pelagic and deep-diving delphinid community in Scottish waters (dolphin data from Plint *et al.* 2023), including the long-finned pilot whales that died in the MSE.

Juvenile and adult long-finned pilot whale occupy distinct trophic niches, primarily driven by differences in $\delta^{15}N$ values (Figure 4). Juvenile animals are most likely affected by a residual nursing signal in tissues with a longer turnover time (e.g. skin). Adult male and female animals showed complete niche overlap, although female animals have a broader range of $\delta^{13}C$ and $\delta^{34}S$ values. This may suggest intraspecific sex-based differences in spatial use of habitat. However, this may simply be a sample size effect (i.e. more female than male animals in this sample set). The wide range of $\delta^{13}C$ values across the sample set indicates broad spatial (across depth and/or latitude) use of resources, particularly among female animals. This supports the hypothesis that multiple pods with variable travel histories were involved in the MSE.

Liver and skin $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ were used to assess sudden isotopic changes in diet in the days-to-weeks-to-months prior to the stranding event (Figure 6). Protein-based tissues/organs have distinct isotopic offsets due to physiological differences in function and metabolic routing during synthesis (for example in marine mammals, see Smith *et al.* 2021; Clark *et al.* 2019). Liver and skin tissue mean isotopic offsets are reported in Figure 7. Liver $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ was standardized to skin using these offsets (Figure 7). Liver has a rapid cellular turnover rate of 10 to 14 days and represents the most recent isotopic signal integration from diet, while skin has a slower cellular turnover rate, with a half-life of approximately 24.16 ± 8.19 days for carbon and 47.63 ± 19 days for nitrogen (Gimenez *et al.* 2016). Based on this method, the similarity between offset-corrected liver and skin $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ (Figure 7) do not indicate any sudden shifts in diet within the 10 to 14 days leading up to the mass stranding event. Additional time-series dietary modelling will form a major component of A. Kebke's PhD thesis (University of Glasgow).

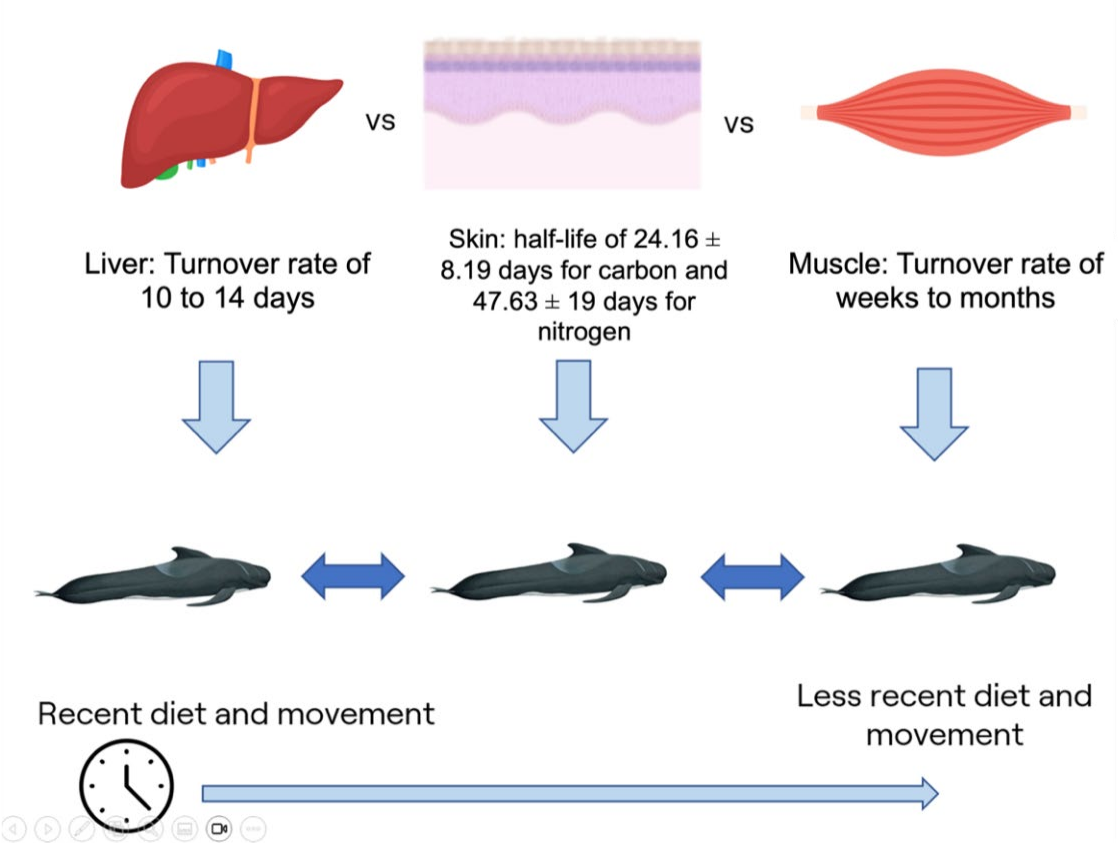

Figure 6. Long-finned pilot whale tissue time-series, where the isotopic composition of liver represent the most recently integrated dietary signal.

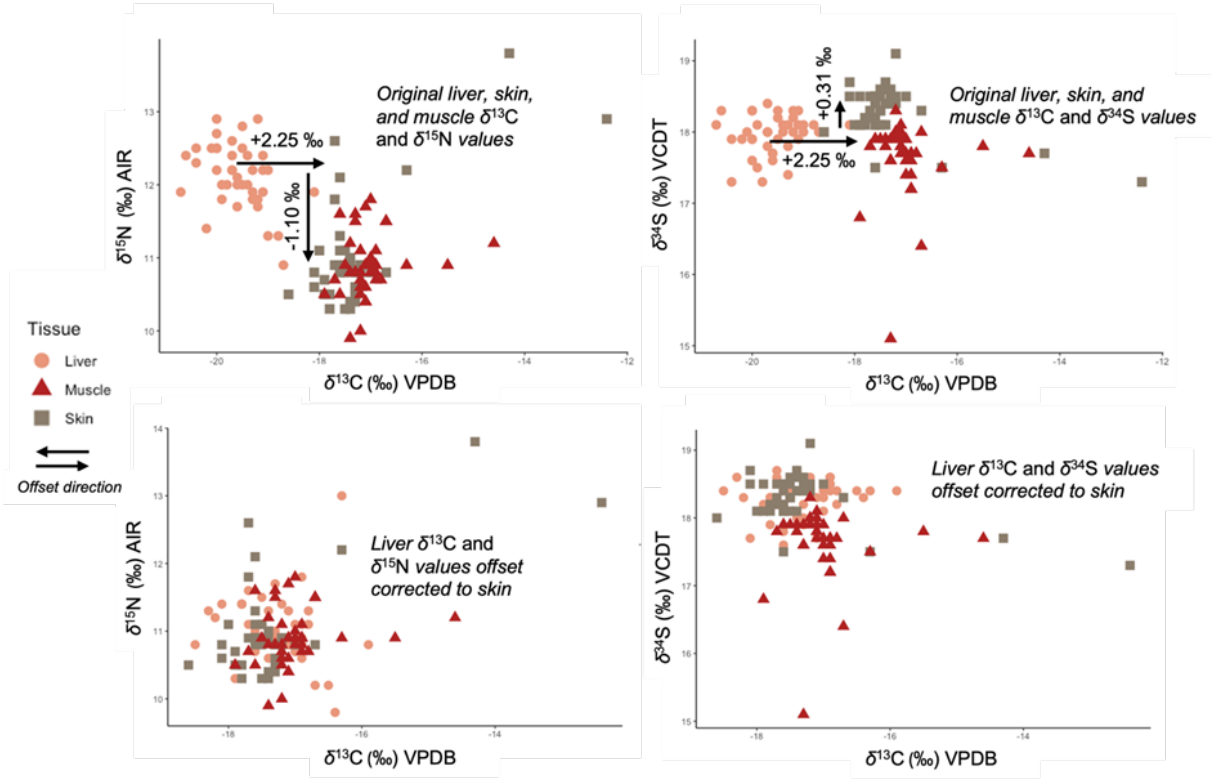

Figure 7. The original and tissue offset corrected isotopic composition of long-finned pilot whale liver (showing shorter-term diet signal integration) and skin (showing longer-term diet signal integration) samples from the MSE.

2.3.2. Long-finned pilot whale blubber fatty acid composition

Long-finned pilot whale blubber fatty acids were composed of 10 to 24 carbons (medium to very-long chained) saturated (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). As expected for marine mammals, *de novo* synthesized saturated and monounsaturated fatty acids (14:0, 16:0, 18:0, 16:1n-7, 18:1n-9) were the most abundant. As expected in odontocetes (Koopman *et al.* 1996; Strandberg *et al.* 2008), inner and outer blubber layer fatty acid composition was stratified. Saturated fatty acids (SFA) and diet-derived MUFA (20:1 and 22:1) and PUFA were more abundant within the metabolically active inner blubber layer (Table 3; Figure 8).

Diet-derived MUFA (20:1 and 22:1) and PUFA were more abundant within the inner blubber layer of MSE animals deemed in “good” body condition (based on blubber and muscle depth measurements) at time of post-mortem examination, than in single stranded animals in “poor” body condition (Figure 9). PUFA are essential to maintaining endocrine and nervous system function yet cannot be internally biosynthesized and must be attained from diet. The abundance of diet-derived PUFA within the inner blubber is an indicator of foraging success and body condition (Plint, PhD thesis, unpublished data). The inner blubber layer of animals involved in the MSE showed higher PUFA levels and did not indicate any signs of extended poor foraging success. As a comparison, single-stranded long-finned pilot whales with thin blubber layer thickness and depleted back muscle deposits showed extremely low levels of diet-derived PUFA within their inner blubber layer (Table 3). The inner blubber ΣPUFA/ΣSFA (which decrease with deteriorating nutritional condition as diet-derived PUFA are utilised and not replaced) indicates that the animals involved in the MSE were in good nutritional condition with no evidence of chronic poor foraging prior to stranding (Table 3).

Table 3. The inner and outer blubber layer fatty acid composition of the MSE animals in comparison with single-stranded animals in poor body condition at time of death (body condition determined based on thin blubber and back muscle layer thickness).

Relative abundance (%)	**2023 MSE inner blubber**	2023 MSE outer blubber	**Single-stranded inner blubber**	Single-stranded outer blubber
ΣSFA	**25**	19	**17**	18
ΣMUFA	**63**	78	**81**	80
ΣPUFA	**12**	3	**2**	2
ΣPUFA/ΣSFA	**0.48**	**0.15**	**0.12**	**0.11**

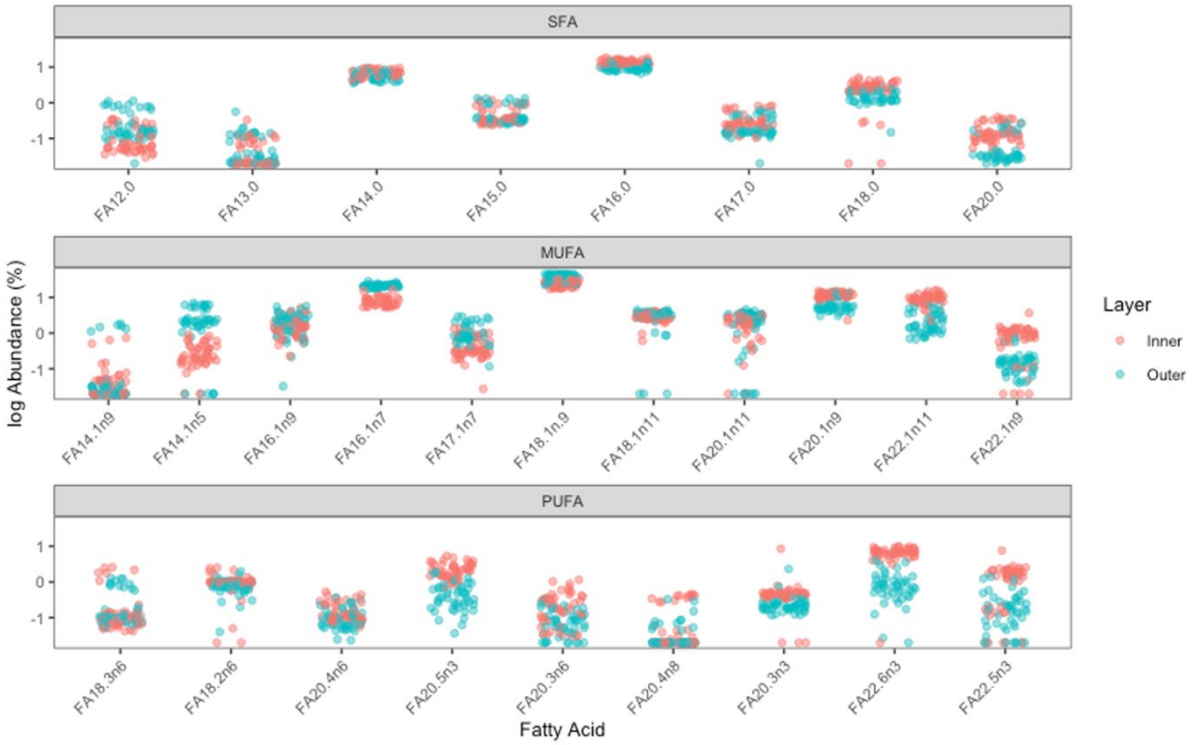

Figure 8. Fatty acid distribution according to blubber layer for juvenile and adult long-finned pilot whales involved in the MSE.

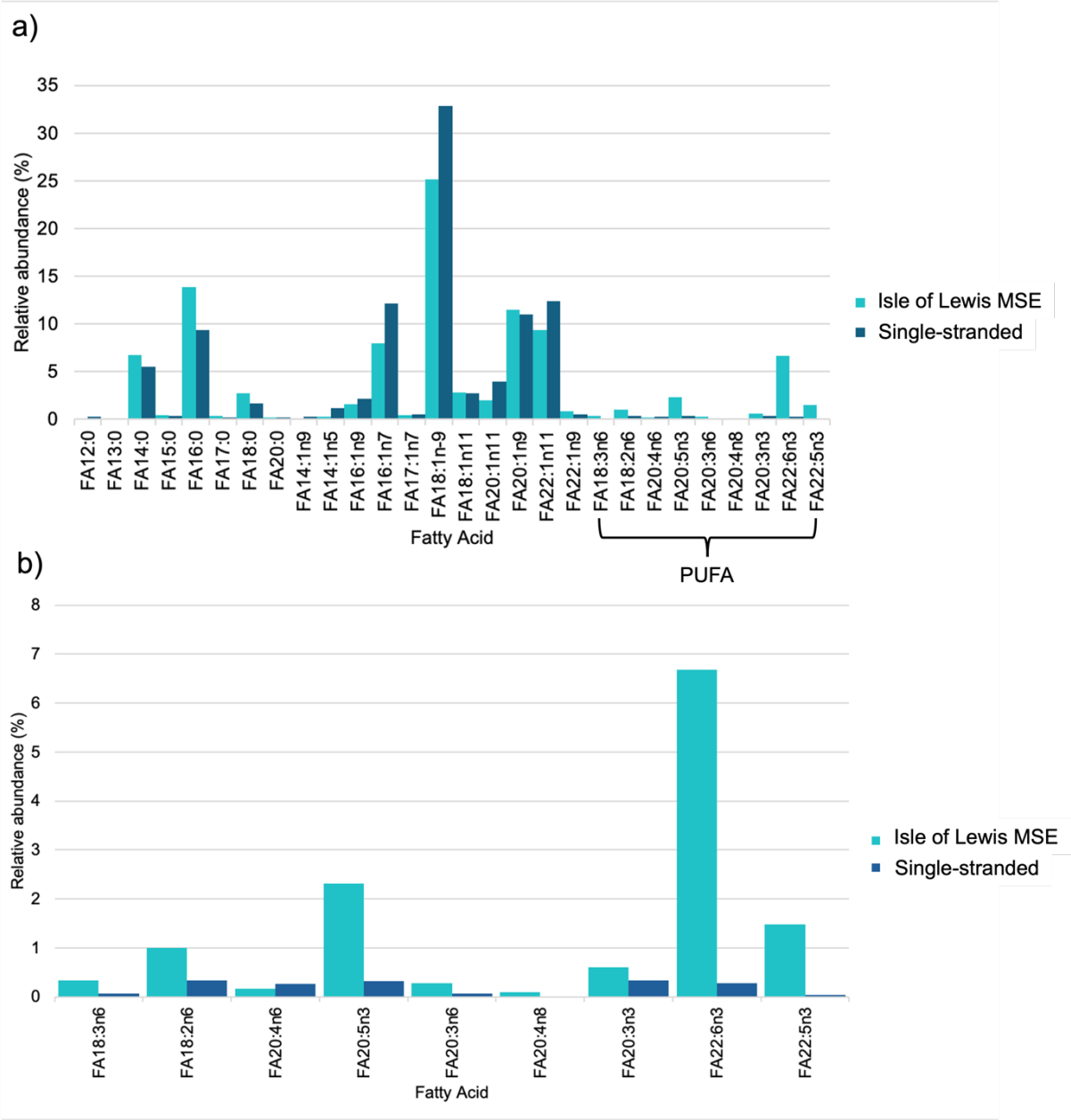

Figure 9. a) Comparison of inner blubber layer fatty acid composition between animals involved in the MSE (deemed in good body condition) and single stranded animals recorded in poor body condition. b) Comparison of inner blubber layer dietary PUFA between MSE and single stranded animals. Animal nutritional condition impacts the abundance of dietary fatty acids within the metabolically active inner blubber layer. Inner blubber diet-derived PUFA levels decrease in animals that are not meeting their energetic demands, as diet-derived PUFA are utilised and not replaced.

3. Genetics (Objective 2)

The second objective is to investigate the genetic relationships and social structure within the Isle of Lewis MSE (i.e. assessing whether the individuals involved were a single cohesive matriline grouping or multiple distinct matrilines that were in association prior to stranding). Additionally, the kinship cohesion hypothesis will be tested to find out if closely related individuals (i.e. mother-juvenile pairs) were in close spatial proximity to each other. This information will elucidate the social dynamics of LFPW and explore the potential role of stress-induced pod aggregation as a contributing factor to mass strandings.

Genetic analysis of these individuals will allow for the gathering of information on kinship and wider population connectivity, and those findings can be further related to morphological and physiological trends. When combined with data from the wider area; insights into population structure, genetic connectivity, and the potential for genetic erosion resulting from the loss of maternal lines can be gathered – a potential critical loss for this long-lived, slow-reproducing species.

3.1. Aims

We used 12 genetic microsatellite markers to generate individual genetic profiles (i.e. genotypes for the LFPWs involved in the Tolsta MSE (and the 2024 Orkney, Sanday MSE: Appendix 2)). These individual genotypes were then used to investigate population differentiation and connectivity compared to previous LFPW samples (*n* = 168), including a captured pod, MSE and Single stranding samples (1995–2020) (collected as part of R. Ball's PhD research, 2023, unpublished data). Furthermore, kinship within the MSE was established to elucidate aspects of relatedness, social structure, and spatial proximity to address the following questions:

Q1. Do the recent MSE show population connectivity or differentiation with previously stranded LFPW across the eastern North Atlantic?

H1. We expect a lack of significant genetic structure among all eastern North Atlantic samples, compatible with them all being derived from a single genetically cohesive population.

Q2. Were multiple pods involved in the MSE?

H2. We expect the MSE to contain multiple family groups. Family group composition is expected to show mother-offspring pairs across multiple female-daughter generations (i.e. an adult female with an adult daughter who also has offspring).

Q3. Were closely related individuals found in close spatial proximity to each other?

H3. We expect that mother-offspring pairs and close relatives will not be in close spatial proximity, indicating disruption to social bonds between LFPW. Additionally, some juveniles may not have mothers present among the deceased individuals.

3.2. Methods

3.2.1. DNA Extraction and Microsatellite Genotyping

DNA (deoxyribonucleic acid) was extracted from 56 LFPW tissue samples (*n* = 36 adults, *n* = 17 juveniles, *n* = 3 foetus) collected from the Tolsta MSE. Extractions were performed using the Qiagen DNeasy Blood & Tissue Kit, following the manufacturer's protocol.

Each sample was genotyped using 12 microsatellite primers, newly developed at Aberystwyth University by Dr Niall McKeown. The primer pairs were specifically designed for LFPW to target tetra-nucleotide repeats on different chromosomes (Table 4). The forward primer of each pair was labelled with a fluorescent dye to allow simultaneous sequencing (multiplex) of loci (i.e. microsatellite), even if they overlapped in the size range of fragment lengths; in this instance, the loci were combined into three multiplex panels of four primers in each (Table 4).

3.2.2. PCR Amplification

Polymerase Chain Reaction (PCR) amplifications were carried out in a 10 µL reaction volume, for each of the 12 markers, amplifying them one-at-a-time. Reactions consisted of 5 pmol of each forward and reverse primer, 0.2 U of Taq DNA polymerase (ThermoFisher Inc), 1x supplied PCR Buffer (1.5 mM $MgCL_2$ & 0.2 mM dNTPs), and DNA Sample**.**

PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min then, 35 cycles of: 95°C for 30 seconds (denaturation), 52°C for 30 seconds (annealing), 72°C for 30 seconds (extension), and a final extension step of 72°C for two minutes.

3.2.2.1. Genotyping and Fragment Analysis

For each individual sample, undiluted PCR products for each marker were pooled into three multiplex panels, as detailed in Table 4 and subsequently diluted 1:20. Multiplex panels were then denatured using formamide and analysed using capillary electrophoresis on an Applied Biosystems 3500 sequencer at Aberystwyth University, with the internal size standard LIZ250 (red) (Further details in Appendix 1). Alleles were scored (genotyped) using Peak Scanner software (Applied Biosystems Inc.).

3.2.2.2. Quality control

To assess genotyping accuracy, repeat amplification and genotyping were performed on 18% of samples across all 12 loci. Microchecker (Van Oosterhout *et al.* 2004) was used to check for the presence of null alleles (i.e. alleles that do not amplify and result in spurious homozygous genotypes), large allele dropout (i.e. amplification of the smaller of two alleles during PCR due to competition in the reaction) and deviations from neutral expectations for individual loci based on the Hardy-Weinberg Equilibrium (HWE) (further details in Appendix 1).

Table 4. Microsatellite markers with tetra-nucleotide repeats, specific to LFPW, indicating which chromosome the marker was found on. Multiplex panel groupings and which fluorescent dye the locus was labelled with (colour indicates the colour of the dye: FAM is blue; VIC is green; NED is black; PET is red). The observed number of alleles across all of the samples and Size Range = the observed range in fragment size across all samples (*n* = 303).

Locus	Multiplex	Fluorescent dye	Observed no of alleles	Size Range
Chromosome 1	A	FAM	10	184 – 220
Chromosome 5	A	VIC	7	231 – 343
Chromosome 6	A	NED	8	222 – 254
Chromosome 7	A	PET	10	208 – 246
Chromosome 8	B	FAM	9	157 – 191
Chromosome 9	B	VIC	7	142 – 166
Chromosome 11	B	NED	6	222 – 246
Chromosome 16	B	PET	7	183 – 207
Chromosome 17	C	FAM	8	192 – 220
Chromosome 20	C	VIC	6	222 – 242
Chromosome 21	C	NED	6	149 – 169
Chromosome X	C	PET	7	147 – 171

3.2.3. Data Analysis

3.2.3.1. Population Genetic Analysis

To set more context for the focal MSE, the samples were combined with LFPW samples (*n* = 168) from R. Ball's PhD research, including a captured pod from the Faroes, MSEs and Single stranding samples from Scotland and Ireland, hence forth referred to as Northeastern Atlantic samples (NeA). The Sanday 2024 MSE samples (Appendix 2) were also included in the NeA samples. Each MSE and the collective group of single strandings are considered "populations", for the purpose of the genetic analyses. The programmes genepop (Rousset 2008), GenAlEx (Peakall & Smouse 2005), and HP-Rare (Kalinowski 2005) were used to analyse population structure based on the individual genotypes for the NeA samples by assessing summary statistics within populations:

- Observed heterozygosity (Ho): the percentage of loci in a population that is heterozygous, calculated for each locus by dividing the total number of heterozygotes by the sample size.
- Expected heterozygosity (He): the average proportion of heterozygous genotypes expected at a locus for a population under HWE (calculated as one minus the sun of the squared allele frequencies at a given locus).
- F_{IS}: an inbreeding coefficient, based on the difference between H_O and H_E.
- Number of different alleles in each population (Na) and rarified Na: the process of rarefying the data to a common sample size to get a more accurate estimate of the number of alleles if all populations were the same size.
- Number of Private alleles (i.e. alleles found only in a single population) and relative proportion of private alleles using the rarefying method.

Deviations from the assumptions of HWE, were tested in genepop (Rousset 2008), which could suggest non-random mating (inbreeding) if there is an excess of homozygotes, compared to those expected based on the allele frequencies. Alternatively, if there is an excess of heterozygotes then random mating (avoidance of relatives) or hybridisation between genetically distinct populations might be occurring. Finally, if there is an overall deviation from neutral expectations then there could be hidden population structure among the samples.

Pairwise assessment of differentiation between populations based on F_{ST} with 10,000 permutations was calculated in *hierfstat* (Goudet 2005)

A Principle Components Analysis (PCA) of the distribution of genetic variation across samples in relation to their population of origin was produced in *adegenet* (Jombart 2008) for the samples that originated from Scotland.

3.2.3.2. Relatedness Analysis

To assess the kinship and relatedness of the LFPW in the MSE, three methods were used to determine pairwise relatedness and sibling-ship reconstruction. The results of those methods were then combined to generate consensus parent-offspring dyads and groups of kin. For six of the samples, DNA was extracted from both the mother and foetus, enabling ground truthing of the genetic relatedness analyses. All individuals from the MSE were treated as potential offspring to test for parent-offspring dyads between adult individuals. All adult females within the MSE were treated as potential mothers. Adult females were then ranked by size, to exclude smaller females from being considered as the parent in a dyad with much larger females.

ML-R_{ELATE} (Kalinowski, Wagner & Taper 2006) was used to calculate pairwise relatedness using the Kalinowski dyadic estimator. Maximum-likelihood familial relationships were estimated with the relatedness coefficient (r) thresholds:

- $r \approx 0.5$: First degree relationships (Parent-Offspring, Full-Sibling).
- $r \approx 0.25$: Second degree relationships (Half-Siblings, Grandparent-Grandchild).

C_{ERVUS} (Kalinowski, Taper, & Marshall, 2010) was used to assess the likelihood ratio of parent-offspring dyads, accounting for the proportion of candidate parents sampled. Confidence levels of 95% and 85% for the assigned parent-offspring dyads were used as cutoff values for the LOD scores to reduce misclassification.

Finally, COLONY (Jones & Wang 2010) was used to reconstruct the siblingship between individuals within the MSE. The full-likelihood approach was used which tests for both parent-offspring and sibling dyads among the whole group. Parent-offspring dyads were retained if they were found in two of the three methods described above.

As part of the sibling-ship reconstruction, COLONY infers mothers for individuals that are not assigned to a mother from the sample set. The inferred mothers were then used to test for sub-structure among individuals with the same inferred mother. If the probability of those individuals having the same inferred mother was greater than 0.5 then that group of individuals was classed as a potential genetic unit. ML-R_{ELATE} was then used to test that second-degree relationships were the most likely relationship between individuals within the genetic unit (See Appendix 1).

3.2.3.3. Relatedness visualisation

To visualize genetic relationships within the Tolsta MSE a consensus relatedness matrix of the first and second-degree relationships was constructed from, the ML-R_{ELATE}, C_{ERVUS} and COLONY results. This matrix was then used to draw a Fruchterman-Reingold force-directed weighted network using R packages *igraph* (Csardi & Nepusz 2006) and *popgraph* (Dyer, Nason & Garrick 2010).

To investigate the spatial proximity of closely related individuals on the beach at Tolsta, a map of the stranding event was drawn in *ggplot2* (Wickham 2016) using the individual locations taken upon sampling. The position of mother-juvenile dyads was plotted and arrows (pointing to the offspring) used to link the dyads across the beach. Additionally, the maternal clusters identified in COLONY and ML-R_{ELATE} were colour coded and plotted to show the different maternal families present within the Tolsta MSE and their distribution on the beach.

3.3. Results

3.3.1. Microsatellite dataset

Microsatellites were amplified successfully for all 56 individuals from the Isle of Lewis 2023 event, 93% of individuals had all 12 microsatellites genotyped and all were scored with no fewer than 9 microsatellites. Genotyping error was estimated to be 0.07 per locus based on replication. Upon integration with R. Ball's PhD samples (and Sanday: Appendix 2), 303 samples were included in the NeA data set. None of the 12 loci showed evidence of null alleles or allelic drop out. HWE analysis of locus by locus, showed no significant deviation from equilibrium.

3.3.2. Population structure

The Tolsta MSE showed no difference between He and Ho and no evidence of a deficiency of heterozygotes, Table 5 (which would have suggested inbreeding, if F_{IS} was also large and positive). Within the collection of individuals 4 private alleles were detected, these had not been found before in all the samples from the wider eastern North Atlantic region.

Table 5. Summary diversity statistics for the NeA samples: n = sample size, Na = mean number of alleles per locus, rNa = rarefied mean number of alleles per locus to account for sample size, Ho = observed heterozygosity, He = expected heterozygosity, F_{IS} = allele frequency based correlation between Ho & He, HWE = Hardy-Weinburgh probability test p-values (in parenthesis is the p-value result for heterozygosity deficienty for the Scottish Single samples), Fisher's = Fisher's exact test p-value for heterozygosity excess, P.A = number of Private Alleles, P.Ar = Private Allele richness averaged over loci to account for sample size. Significant values are in bold.

Group	n	Na	rNa	Ho	He	F_{IS}	HWE	Fisher's	P.A	P.Ar
Tolsta	57	6.4	4.1	0.73	0.73	0.009	0.751	0.904	4	0.34
Sanday	78	6.3	4.2	0.77	0.75	-0.024	0.913	0.184	2	0.09
Rutland	32	5.7	4.0	0.73	0.71	-0.012	0.478	0.357	2	0.17
Durness	13	5.0	3.8	0.72	0.66	-0.059	0.810	**0.030**	2	0.17
Pittenweem	21	5.2	3.9	0.74	0.72	-0.007	0.817	0.270	0	0.00
Falcarragh	6	3.9	3.4	0.72	0.61	-0.081	1	0.069	1	0.08
Staffin	8	4.3	3.7	0.75	0.68	-0.033	0.931	0.368	0	0.00

Group	n	Na	rNa	Ho	He	F_{IS}	HWE	Fisher's	P.A	P.Ar
Faroes	26	5.3	3.9	0.73	0.68	-0.044	0.936	0.191	1	0.09
Lochboisdale	8	4.8	4.0	0.78	0.69	-0.060	0.994	0.073	1	0.08
Scottish Single	24	5.9	4.2	0.74	0.74	0.043	**0.009** (0.052)	0.949	2	0.25
Irish Single	31	5.7	4.1	0.72	0.72	0.012	0.189	0.953	0	0.00

Pairwise F_{ST} between each grouping in the NeA sample set shows low values but significant differences for the Tolsta MSE with four other MSEs that have occurred (Table 6). This is likely due to the different family groupings present in the different MSEs that are unrelated to each other.

PCA analysis of only the Scottish samples (Figure 10) shows the genetic variation across samples in relation to their MSE or Single stranding of origin. No distinct clustering is observed from any of the MSEs or the Single strandings which correlates with gene flow and mixing among individuals across Scottish waters. Furthermore, the Tolsta MSE does not cluster separately (Figure 10), so this MSE encompassed individuals from across the wider eastern North Atlantic population, that were in association prior to the MSE beginning. This means that gene flow is occurring throughout the wider eastern North Atlantic and these regions are connected.

Table 6. Pairwise F_{ST} values for each population below the diagonal, p-values are above the diagonal, significant values (level = 0.05) are in bold. 1 = Tolsta, 2 = Sanday, 3 = Rutland, 4 = Durness, 5 = Pittenweem, 6 = Falcarragh, 7 = Staffin, 8 = Faroes, 9 = Lochboisdale, 10 = Scottish Single & 11 = Irish Single groupings.

Location	1	2	3	4	5	6	7	8	9	10	11
Tolsta	-	0.000	**0.012**	**0.035**	0.109	**0.016**	**0.005**	0.000	0.181	0.904	0.000
Sanday	0.012	-	0.000	0.748	0.083	**0.009**	0.204	0.000	**0.005**	0.715	0.000
Rutland	0.018	0.032	-	0.000	**0.014**	**0.001**	**0.002**	**0.012**	**0.005**	**0.002**	**0.001**
Durness	0.009	-0.003	0.042	-	0.056	**0.002**	0.100	**0.044**	**0.018**	0.230	**0.007**
Pittenweem	0.012	0.014	0.039	0.0189	-	**0.038**	0.250	**0.025**	**0.046**	0.357	**0.027**
Falcarragh	0.024	0.027	0.068	0.044	0.031	-	0.140	0.000	**0.037**	0.000	0.000
Staffin	0.014	0.003	0.031	0.008	0.009	0.014	-	0.065	**0.003**	0.172	0.000
Faroes	0.020	0.020	0.020	0.010	0.025	0.052	0.009	-	**0.005**	0.518	0.000
Lochboisdale	0.010	0.038	0.062	0.035	0.036	0.039	0.058	0.045	-	**0.029**	**0.011**
Scottish Single	-0.005	-0.002	0.026	0.002	-0.000	0.048	0.004	-0.002	0.028	-	**0.005**
Irish Single	0.028	0.030	0.033	0.018	0.027	0.060	0.049	0.037	0.040	0.017	-

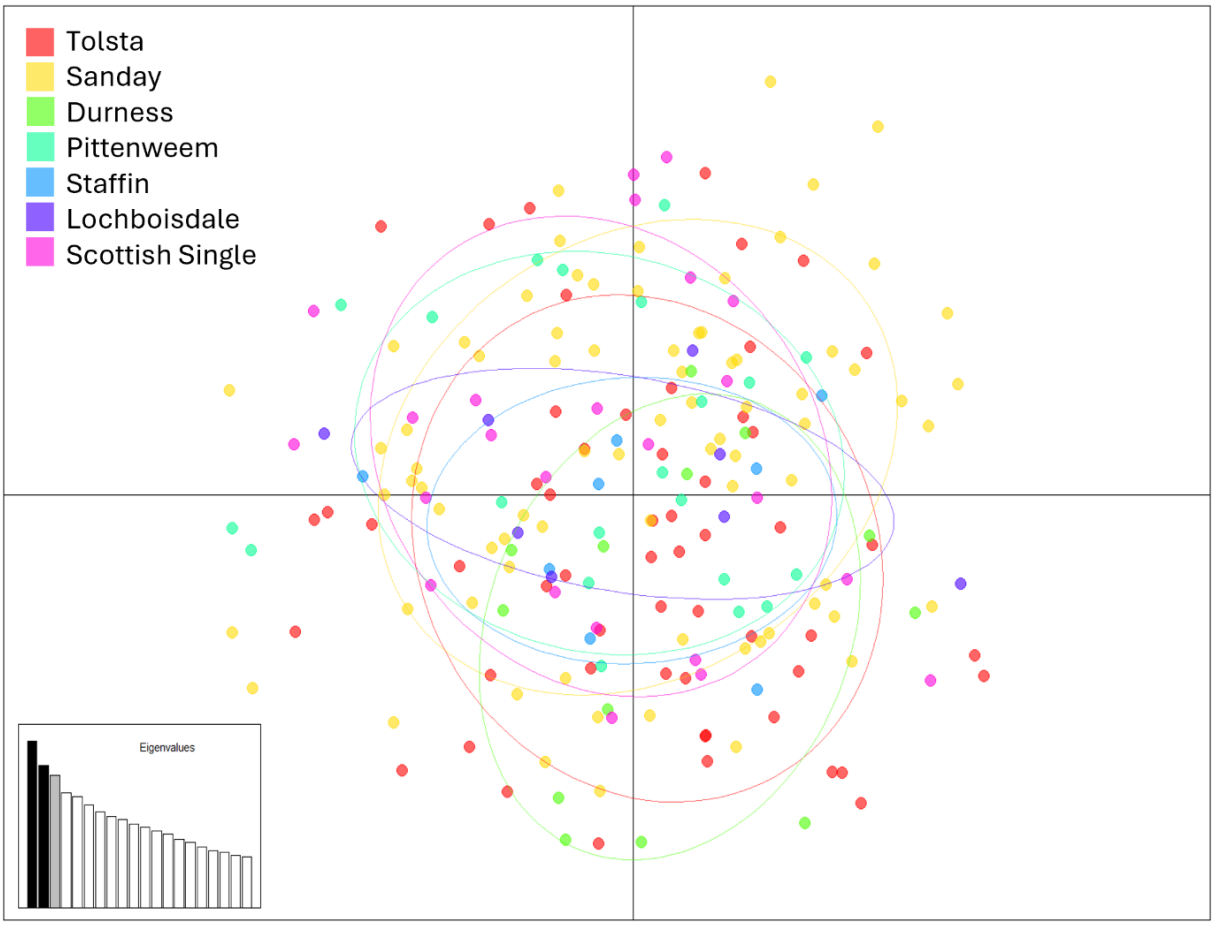

Figure 10. PCA of the genetic variation across the Scottish samples in relation to their "population" of origin.

3.3.3. Kinship Analysis

All 56 individual samples were analysed for their relatedness to every other sampled individual from the Tolsta MSE. For the entire group of 56, the mean pairwise relatedness was 0.022 (i.e. the majority of pairs of individuals were unrelated) indicating that upon further analysis there will potentially be multiple unrelated pods present within this MSE.

The consensus relationships between individuals from the ML-RELATE, CERVUS and COLONY results found there were 22 parent-offspring dyads among the 56 individuals; there were 17 mother-offspring dyads (adult and juvenile offspring) and five suspected father-offspring dyads (adult offspring only). To visualise these connections between individuals Fruchterman-Reingold force-directed network was drawn; the relationships in the second-degree cutoff are shown in Figure 11a. In Figure 11b., only the first-degree relationships are retained to show the distinct close relationships and the individuals with no first-degree relationships. All these individuals have at-least one first or second-degree relationship connecting them to another individual within the MSE. Individual 06, an adult male, is the least connected individual; the first-degree parent-offspring dyad with adult female 29 was his only kinship bond among the 56. This is a suspected father-offspring dyad however, tooth ageing of these individuals would provide confidence to the direction of adult-to-adult parental dyads. Tooth ageing allows for the gestation time to be accounted for in females and accounts for length differences between males and females that may mask their true age (Eichenberger *et al.* 2025; Ford *et al.* 2011). As male LFPW are sexually dimorphic, a male that is longer than an adult female may not be older and therefore might not be the

father. This could be the case for all paternal dyads in this MSE, which is why they are referred to as suspected paternity.

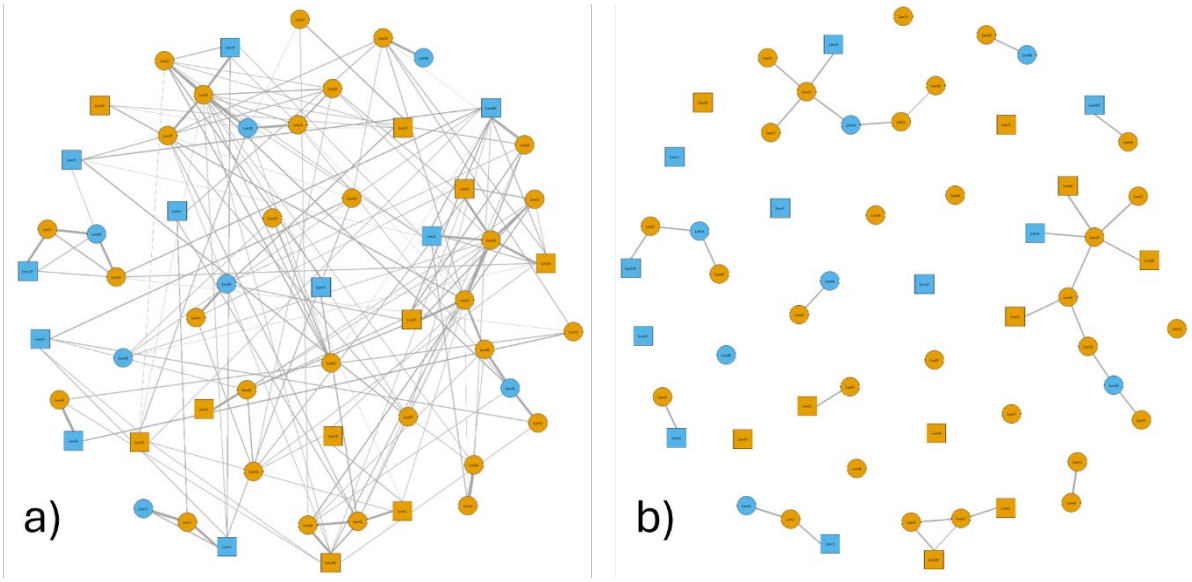

Figure 11. Kinship networks within the Tolsta MSE, adult individuals are represented with a circle, juveniles with a square, males are blue and females yellow in colour. a) shows the second-degree relationships (r = 0.25+, half-siblings & grandparent) and first-degree relationships, the thickness of the connecting lines is indicative of the r-value (i.e strength of the relationship). b) is a stripped back version of the same network (individuals in the same location) with only the first-degree relationships displayed (r = 0.4+, parent-offspring & full-sibling).

It is easier to determine directionality for mother-offspring dyads between female adults as a shorter female is theoretically younger than a longer female, therefore the longer female is assumed to be the mother. However, tooth ageing would greatly benefit the confidence in adult female parent-offspring dyads also to account for gestation time between offspring.

The mother-juvenile dyads were assigned using the consensus of the three methods and with confidence cutoffs (Table 7). There were 11 mother-juvenile dyads assigned, three of those being the dam-foetus pairings. This means that 53% of the juveniles present on the beach (9 of 17), did not have a mother present. As no adult individuals were refloated during this MSE, this means that these nine missing mothers did not strand as part of this MSE.

Table 7. The consensus mother – juvenile parings found in the Isle of Lewis MSE. All the juvenile individuals within the MSE are listed, then the assigned mother and the maternal cluster that pair is part of. The juveniles without a mother present are then listed and listed again with their maternal cluster assignment. Finally the juveniles with no maternal kin in the MSE are listed.

Juveniles = 17 + 3 foetuses (#F)		Juvenile – Mother pairs + Cluster ID		Juveniles NO mothers	Cluster assignment		Juveniles NO cluster
Male = 10	Female = 10						
11	01	30 – 01	D	18	21	L	18
19	18	17 – 11	F	21	35	E	51
20	21	25 – 19	E	35	45	F	54
31	26	24 – 20	I	45	47	J	
45	33	30 – 26	D	47	48	G	
47	35	30 – 31	D	48	53	H	
51	41	42 – 33	D	51			
54	48	12 – 41	B	53			
27F	53	27 – 27F	A	54			
40F	39F	39 – 39F	B				
		40 – 40F	C				

Using the COLONY sibling-ship reconstruction along with the p-value test for second-degree relationships in ML-R_{ELATE} determined individuals that belong to the same maternal cluster. Within a maternal cluster, individuals all share at least a second-degree relationship to each other that is inherited from their maternal line.

Table 8. Maternal clusters showing which individuals belonged in which maternal grouping, based on combined COLONY and ML-R_{ELATE} analysis methods. The individuals that were not assigned to a maternal cluster are listed together.

Maternal Cluster	Members
A	27, 27F, 44, 49, 15
B	39, 39F, 12, 41, 50, 08
C	40, 40F, 16, 05, 02
D	32, 30, 01, 26, 31, 38, 42, 33
E	25, 19, 22, 37, 28, 35
F	17, 11, 23, 45, 46
G	03, 36, 48
H	34, 04, 53
I	24, 20, 52
J	29, 06, 47
K	43, 14
L	07, 21
No	10, 13, 18, 51, 54

From the nine juveniles that did not have a mother present within the Tolsta MSE, six of them were assigned to a maternal cluster (Table 7 & 8). This means that although no mother was present for these six individuals, they did have second-degree relatives (e.g. half-sibling, grandmother) present.

There were 12 maternal clusters present (Table 8), meaning that this MSE contained multiple pod units of different families that were in association prior to the stranding event beginning. Indeed, more than 12 pod units are represented among the 53 individuals, as five individuals were not assigned a maternal cluster and therefore, likely came from different pod unit that did not strand (Table 8).

3.3.4. Spatial analysis within the MSE

Individual location points were used to analyse the proximity of LFPW to their close relatives on the beach. Figure 12 displays the 11 mother-juvenile dyads (from Table 7) positions on the beach relative to the other individuals present in the MSE. The mother-foetus pairs are not separated and are circled and labelled within Figure 12. The other mother-juvenile pairs are connected with an arrow pointing to the juvenile, labelled with the pairing IDs. There was only one pair that did not have any unrelated individuals between them on the beach, 30–31, which were in very close proximity (Figure 12). Individual 30 also had two other juveniles present within the MSE, 01 who was the furthest away from all other individuals in the top left of Figure 12 and 26 who was closer to her mother, but three adults separated them to the south and 26 was in closer proximity to 25. Mother 25's juvenile was in a very tight grouping with two other juveniles and an unrelated adult female (17) who was the mother to a juvenile (11) located north-west of that grouping. Although no unrelated individuals separate mother-juvenile pairing 12 – 41, the mother is in much closer proximity to 17's juvenile (11).

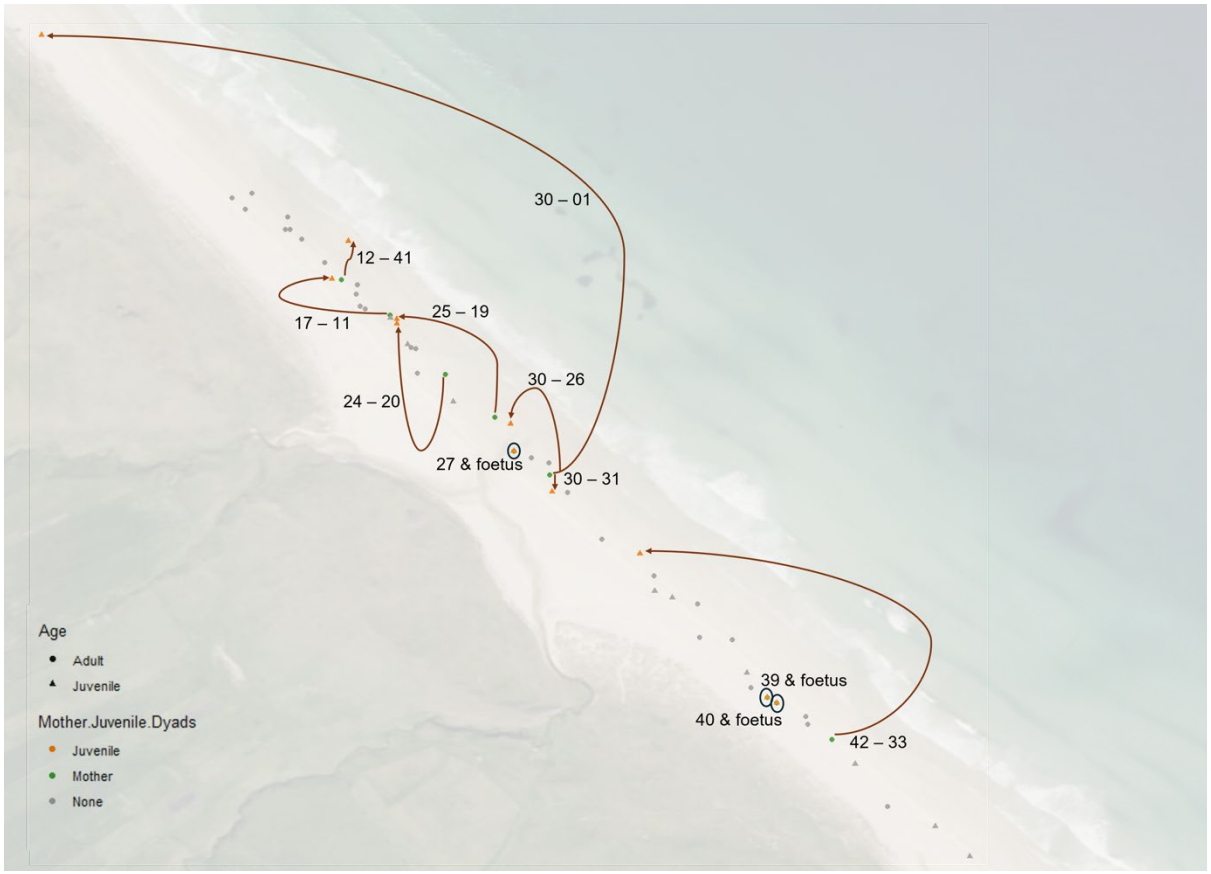

Figure 12. Spatial positions of mother-juvenile dyads on the beach at Tolsta, Isle of Lewis. Adults are represent by a circle and juveniles by a triangle. Brown arrows connect mother and juvenile, with the arrowhead pointing to the juvenile, each arrow is labelled with the individual ID codes. The three females with foetuses are circled and labelled within the plot.

Finally, individual location and maternal cluster assignment was plotted to look for spatial disruption across the whole assemblage of different pod units (Figure 13). Cluster D is the largest maternal cluster and shows the biggest spatial distribution; juvenile 01 at the very top left of the plot, and adult female 42 is the fifth individual in from the bottom right of the plot. Cluster D is also the only cluster that has two adults and a juvenile in direct proximity on the beach (located near the centre of the plot). These individuals are 32-30-31 which are a multi-generational group of mother-adult daughter-juvenile offspring.

The adult female, 40 (labelled in Figure 13), that had the calf stuck in utero does not have any of the other members of her Cluster C nearby, they are all towards the top left of the plot. The two other females (27 & 39) with foetuses also do not have any members of their maternal cluster in their direct vicinity either.

Two (51 & 54) of the three juveniles that were not assigned to a maternal cluster are located at the bottom right of Figure 13 whereas the third (18) is in the very tight grouping with two other juveniles (19 & 20 both have mothers present) and an adult female (17). Four different maternal lines are present in this tight grouping, further highlighting how proximity should not be used as an indication of close kinship.

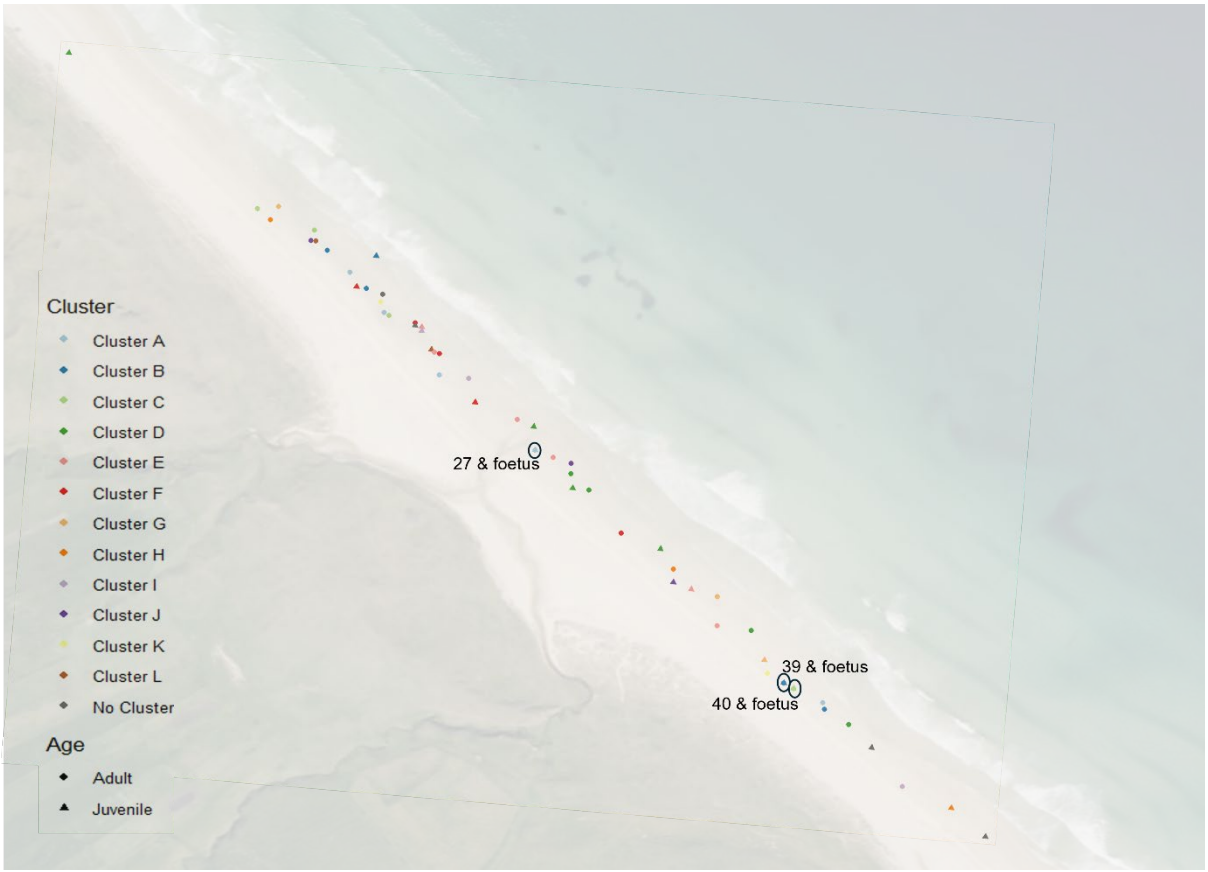

Figure 13. Spatial position of each individual on the beach at Tolsta, Isle of Lewis. Adults are represented by a circle and juveniles by a triangle. Individuals are colour coded according to their assigned maternal cluster. The three dam-foetus pairs are circled and labelled.

3.4. Discussion

3.4.1. Population structure

The Tolsta MSE showed no evidence of significant differentiation to other LFPW samples from the wider eastern North Atlantic region. The MSEs that have occurred over the years, including the Tolsta event, do show a slight excess of heterozygotes, these MSEs could be examples of multigroup associations for breeding purposes. This is further supported by the presence of private alleles which are unique/rare genetic variants and found typically found within a single population among a broader collection of populations (Szpiech & Rosenberg 2011). The lack of differentiation across the eastern North Atlantic region indicates that gene flow is occurring throughout the area. Other studies into cetacean population structure have also reported a lack of population structure, especially among other continental shelf-edge associated species (Banguera-Hinestroza *et al.* 2014; Quérouil *et al.* 2007; Thompson *et al.* 2016).

The significant differences observed between four of the MSEs and the Tolsta MSE in this study is likely a reflection of the different family groupings present within each separate event. Short-finned pilot whales in Hawaiian waters also presented with significant F_{ST} differences between observed social associations, which were formed between individuals with a higher level of genetic relatedness (Van Cise *et al.* 2017).

F_{ST} analyses reported several numerically small but statistically significant pairwise values. Interpreting such patterns against a background of weak structure is notoriously difficult as various sources of errors, such as sampling of kin aggregations (Allendorf & Phelps 1981), and temporal fluctuations (Selkoe & Toonen 2006), may assume a relatively greater importance and lead to false conclusions. Kinship analyses provide a more direct focus on how alleles are shared among groups can complement F_{ST} based analyses (which focus on gene flow among groups).

3.4.2. Kinship

The finding of 53% juveniles without a mother present within the MSE is significant although, not unexpected. Analysis of MSEs in the Southern Hemisphere found that 33–57% of juveniles had no potential mother within their MSE (Oremus *et al.* 2013). This could mean that the mother-offspring bond was separated prior to the MSE beginning or that some individuals present in the at-sea pod complex of LFPW did not strand with the individuals that ended up on the beach. There were no detailed (e.g. number of individuals, behaviour, direction of swimming) reports of LFPW sightings in the area prior to the stranding, although they may have been seen, so the potential degree of temporal separation between stranded offspring and their missing mothers is unknown.

Alloparental care is well documented in cetacean species (Aubin, Michaud & Wal 2023; Gero, Gordon & Whitehead 2015; Sarano *et al.* 2021; Wright *et al.* 2016) and prevalent among individuals from the same matriline. Although it is currently not documented in the literature for LFPW, sperm whales change their dive synchrony to allow different groups of adults to feed at depths out of range for the juveniles, leaving the juveniles in surface waters with the others (Rendell *et al.* 2019). LFPW have been observed providing alloparental care to juveniles (Augusto *et al.* 2017a) and therefore, like other cetaceans this likely occurs between close kin. However, alloparental behaviours were also observed between pod units that had weak social associations (Augusto *et al.* 2017a) but as no genetic studies were done in conjunction with the behavioural observations it is unclear if relatedness was a driver of these interactions or not. While speculative at this time, the juveniles with no first-degree relationships but a maternal cluster and the three juveniles with close relatives present, could be an example of LFPWs providing alloparental care during an infrequent social association. Future work combining relatedness analysis with behavioural observations would be needed to confirm that theory.

The spatial analysis (Figure 12) shows that mother-juvenile pairings are not consistently found near each other, disproving the kinship cohesion hypothesis in the Northern Hemisphere, as well as in the Southern Hemisphere (Oremus *et al.* 2013). If refloating had been possible for this MSE and if juveniles had been refloated with their nearest adult female, assuming close kinship, this may have contributed to re-stranding as unrelated adults and juveniles would have been refloated together. Within the literature there are no case studies of MSE refloat attempts that have also categorised kinship between individuals, future work sampling individuals before refloating attempts begin would be needed to provide an answer. However, reports from previous MSEs in Scotland indicate that refloating juveniles and corralling them in deeper water before refloating the adults resulted in a lower number of immediate restrandings than when individuals were refloated one-at-time (Kyle of Durness MSE vs Pittenweem MSE investigation reports SMASS).

With no prior knowledge of the initial individual that became stranded or the timeframe in which the individuals ended up on the beach, the chronology of the stranding event is not elucidated from genetic analysis. Only one maternal cluster shows some correlation of genetic and location closeness, yet that is only applicable to some of the maternal cluster members; other mother-offspring dyads within that cluster D are far apart from one-another.

4. Genetic and Isotope Combined Analysis (Objective 3)

The third objective is to combine social structure analysis and the stable isotope data to infer spatial differences in resource use among family groups (pods units) prior to their involvement in the MSE. Combining kinship analysis (i.e. maternal cluster as a proxy for pod and social organisation) and the stable isotope data allows us to assess the robustness of maternal cluster assignment

Identifying isotopic differences between groups will help us understand individual pod foraging history and test the hypothesis that stress-induced pod aggregation occurred prior to the MSE. If the stable isotopes are not consistent between individuals within a maternal cluster, then the maternal cluster may not be a true representation of at sea pod unit organisation. Identifying isotopic differences between groups will help us understand individual pod foraging history and test the hypothesis that stress-induced pod aggregation occurred prior to the MSE.

4.1. Methods

The maternal cluster assignment from the second objective (section 4), was used along with the $\delta^{13}C$ and $\delta^{34}S$ isotope signature to test for congruence of isotope signatures within individuals from the same maternal cluster and differences between different maternal clusters.

Seven genotyped individuals did not have isotope signatures and were excluded from the combined analysis. Most maternal clusters retained two or more members; this allows for testing of within group cohesiveness (i.e. are the isotope signatures similar between individuals). The exception was Cluster L which only had one individual left in the cluster, within group similarity was therefore not possible for this cluster. Three of the samples without isotope signatures were the foetuses, the signatures for these samples would have been related to their mothers, so their loss did not impact within or between group analysis. Individual 10 was removed from the 'No Cluster' grouping which retained four individuals after his removal. However, as the only adult male within the 'No Cluster' grouping it would have been interesting to see how his isotope signatures differed to the adult female and two juveniles that presumably all came from different pod units.

Maternal cluster assignment was used to investigate the relationship between relatedness and isotopic signature. A two-way analysis of variance (ANOVA) was conducted in R comparing Cluster, Sex and either $\delta^{13}C$ or $\delta^{34}S$ signature. The effects of both Cluster and Sex were tested as Sex differences were observed in the isotope signatures.

4.2. Results and Interpretations

4.2.1. Carbon Isotope and Maternal Cluster

There was no overlap between the $\delta^{13}C$ signatures of Cluster A*, B* and C*. This shows that the three clusters (i.e. pod units) were distinct from each other, both genetically and their respective $\delta^{13}C$ signatures. Clusters A, B and C each contained a pregnant female the consistent $\delta^{13}C$ signatures between the individuals within each cluster shows that these maternally related individuals were a cohesive pod unit. Establishing a cohesive pot unit genetically and with the $\delta^{13}C$ signature, shows that these three pod units were foraging in different locations prior to the MSE (Figure 14).

In Cluster F, there is an adult male-adult female parent-offspring dyad (23–17). Although the adult male is longer in body length than the adult female, without tooth-aging we cannot say

for certain if he is the offspring or the father. However, the overlapping $\delta^{13}C$ signatures for the males and females within Cluster F indicate that these individuals were foraging together, therefore the adult male (23) is more likely to be the offspring of 17, remaining in his natal pod unit. Early studies on the Faroe Grinds showed that adult male LFPWs stay within their natal pod and do not father individuals within the pod in which they are found (Amos, *et al.* 1993). The $\delta^{13}C$ signatures can, therefore, provide further indication that adult males remain with their natal pod. Tooth-aging would assist in the certainty of this conclusion to ensure that this is not a unique record of a father being found in the same pod as his offspring.

Cluster A has a substantially different $\delta^{13}C$ signature from all other clusters present in the MSE and the 'No Cluster' individuals (Figure 14). This shows that Cluster A was foraging in a different location to all the other pod units in the MSE.

The overlapping $\delta^{13}C$ signatures for the females and males within Cluster E and F indicated that all the individuals within these respective clusters (i.e. pod units) were foraging together in the same location prior to the MSE.

Cluster H contained three second-degree related individuals, two juveniles (female and male) and one adult female. The adult female did not have any isotope signature values and was not included in the analysis. The male and female juveniles remaining in the cluster appear to have different $\delta^{13}C$ signature values, making them appear as the least cohesive cluster however, with one missing member it is uncertain if they are truly that separate.

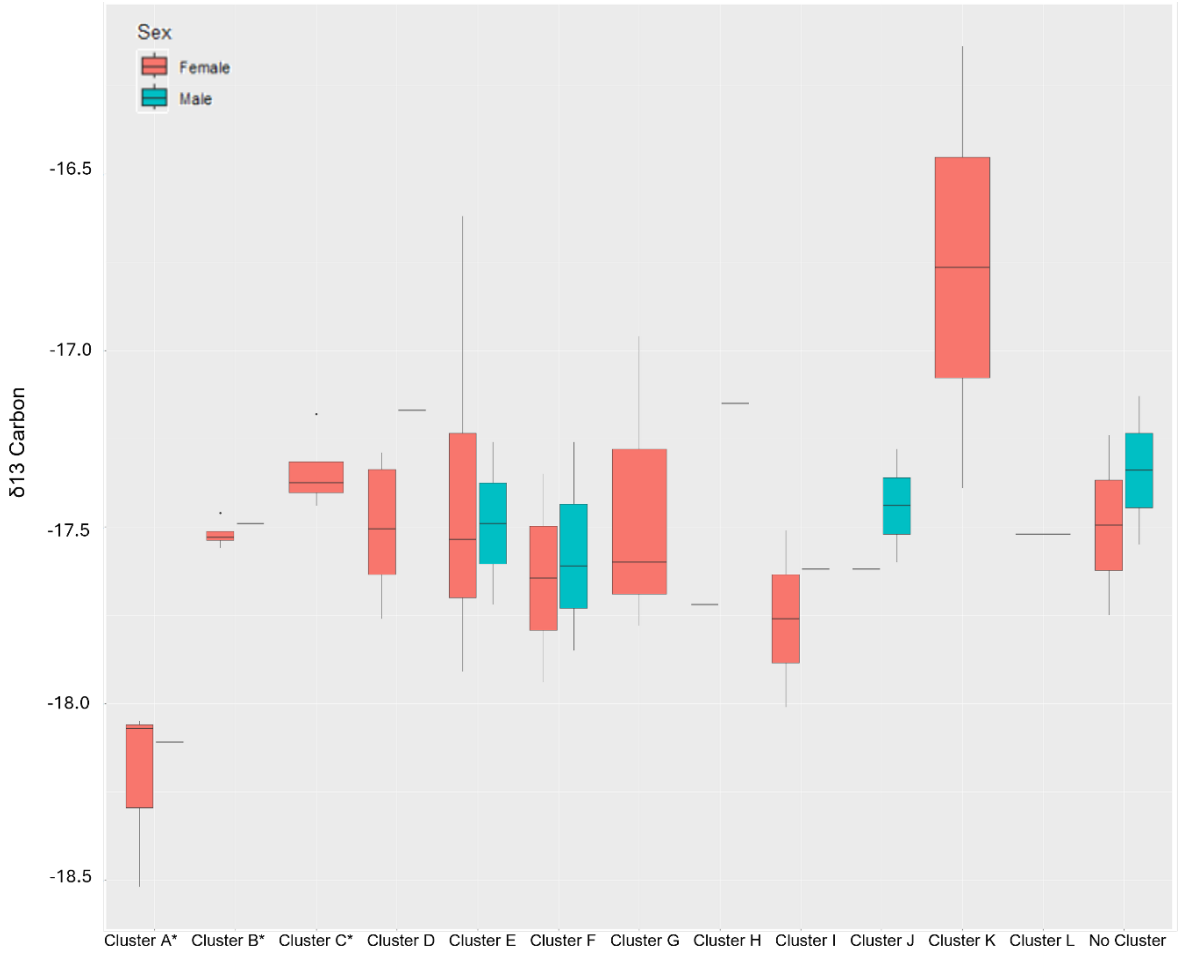

Figure 14. Box plot of maternal cluster and sex against $\delta^{13}C$. The clusters with the three pregnant females in are indicated with an asterix after their Cluster ID letter.

The two-way ANOVA shows that the maternal cluster (pod unit) has a significant impact on the $\delta^{13}C$ signature a pod unit has (Table 9), (i.e. individuals within a pod unit forage in the same location). Sex and Maternal Cluster and Sex together had no significant correlation with $\delta^{13}C$ signature (Table 9).

Table 9. Two-way ANOVA results for $\delta^{13}C$, significant values in bold.

	df	Sum sq	Mean sq	F value	Pr(>F)
Cluster	12	3.342	0.278	2.281	**0.037**
Sex	1	0.130	0.130	1.068	0.311
Cluster:Sex	8	0.212	0.026	0.217	0.985

4.2.2. Sulphur Isotope and Maternal Cluster

Maternal clusters A*, B*, and C* (all clusters containing pregnant females and their second-degree close relatives) also show differences in $\delta^{34}S$ which relates to their respective foraging histories and spatial use of resources (Figure 15). These different pod units have different matrilines, and $\delta^{13}C$ and $\delta^{34}S$ signatures supports the concept that they foraged in different places and had their own travel histories as separate pod units, before coming together in the MSE.

For the individuals in the 'No Cluster' group, the separate $\delta^{34}S$ signatures between the males and females add further weight to these individuals being from separate pod units as they have historically been in different foraging locations (Figure 15).

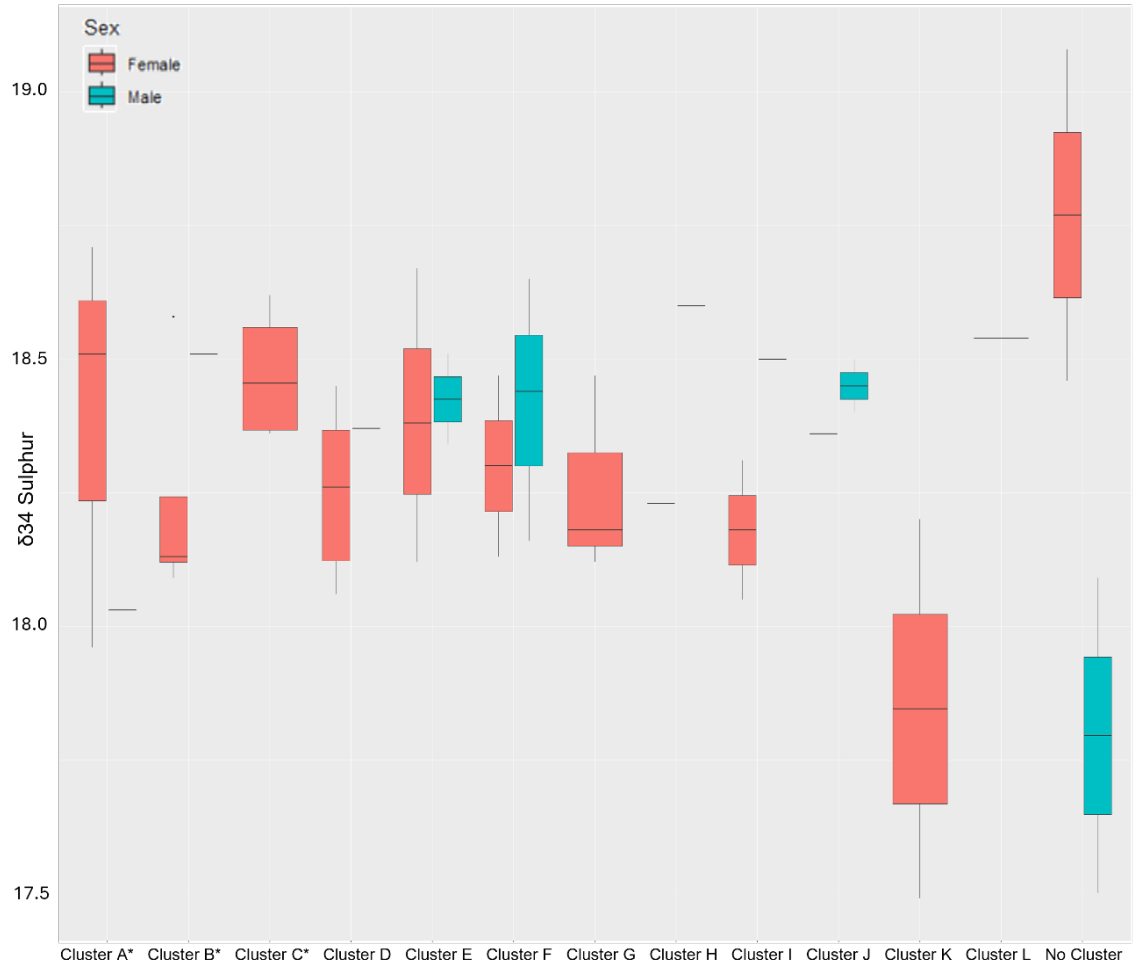

Figure 15. Box plot of maternal cluster and sex against $\delta^{34}S$. The maternal clusters containing the three pregnant females are indicated with an Asterix after the cluster ID letter.

The two-way ANOVA shows that the combined effect of maternal cluster and sex does significantly impact group $\delta^{34}S$ signature (Table 10). Investigations as to why the combination of maternal cluster and sex impacts $\delta^{34}S$ signature is beyond the scope of this report. However, it may be related to slight differences in foraging strategy (which may in turn be based on size differences between males and females) or separate social associations between males and females at different times. Future multidisciplinary work would be needed to investigate this further.

Table 10. Two-way ANOVA results for $\delta^{34}S$, significant values in bold.

	df	Sum sq	Mean sq	F value	Pr(>F)
Cluster	12	0.737	0.061	0.961	0.506
Sex	1	0.01	0.01	0.153	0.699
Cluster:Sex	8	1.27	0.159	2.491	**0.036**

5. Conclusions

5.1. Molecular foraging ecology

- Long-finned pilot whales occupy a distinct isotopic foraging niche within the Northeast Atlantic pelagic delphinid community, with little direct overlap with other delphinid species.

- The MSE animals did not appear to show signs of a sudden dietary shift that could equate to a sudden change in foraging depth/habitat type. This was contrary to our hypothesis that these animals may have been exploiting prey resources in shallower waters prior to stranding.

- The MSE animals had a similar isotopic signal to animals previously stranded in Scotland and Iceland, which supports the hypothesis that they were resident to the northeast Atlantic waters around Scotland

- The MSE animals did not show signs of poor nutritional condition that could indicate they were suffering from chronic poor foraging success.

5.2. Genetic analysis

- LFPW in eastern North Atlantic waters do not show any evidence of heterozygosity deficiency or inbreeding, indicating that these individuals collectively represent a well-connected population with strong gene flow and no population structure by location across the region.

- Comparisons of the Tolsta MSE to the samples from the wider eastern North Atlantic revealed that a similar number of private alleles (when accounting for sample size differences) was found in this one assembly of individuals, as was found in the 20-year period of single strandings.

- The Tolsta MSE contained at least 12 multiple pod units (families) of LFPW. Despite high relatedness within maternal clusters the overall relatedness of individuals in this event was low. The presence of multiple unrelated pod units and the fact that the MSE occurred in the month of July, which is within LFPW breeding season, indicates this large assembly of LFPW were likely in a multigroup association for breeding purposes prior to the MSE (de Stephanis *et al.* 2008).

- The Tolsta MSE had a high proportion of juveniles with missing mothers (53%), but. six of the nine juveniles missing mothers could be assigned to a maternal cluster, which may be a source of alloparental care. If the stressor instigating the MSE occurred during an alloparental care event, that could be an explanation for so many missing mothers. Future work would need to combine behavioural observations with kinship analysis to characterise who is providing alloparental care and at what scale.

- Spatial disruption of close kin was evident, with juvenile-mother pairs separated across the stranding location. Although some close groupings in space and relatedness were observed, it must not be assumed that spatial proximity is an indication of kinship; these groupings were in the minority.

5.3. Concluding summary

The LFPW involved in the MSE had been foraging well, were in good nutritional condition and showed no abrupt changes to their diet prior to stranding. It is unlikely that the pursuit of prey in unfamiliar shallow waters led to stranding. Multiple pod units aggregated prior to the MSE. Pod units contained (at minimum) between 2 to 8 closely related individuals and relatedness between these separate pod units was low.

Spatial disruption of close relatives was prevalent throughout the stranding location; the majority of mother-juvenile pairings were separated on the beach and individuals from different pod units were mixed together.

The presence of multiple unrelated pod units, in good nutritional health indicates that this may have been a multigroup association for breeding purposes. The combined isotope and genetic analysis revealed that pod units showed distinct spatial isotope signatures ($\delta^{13}C$ and $\delta^{34}S$), furthering the evidence for multigroup association prior to this MSE. Distinction of pod units using the multidisciplinary approach, indicates the intriguing possibility of using dietary signals to identify pod clusters in future MSEs, and shows the value of combining different methodological approaches.

These findings support the hypothesis that long-finned pilot whale congregate in offshore waters around northwest Scotland (June–August) and use these waters as a breeding or calving ground. The presence of juvenile animals without mothers or closely related pod members indicates that this mass stranding event (large as it was) may not have included all animals from all pods that were present in the immediate area that day. Marine management bodies should take these regional LFPW aggregations into account when assessing potential sources of anthropogenic disturbance to deep-diving marine mammals in Scottish waters.

LFPW have exhibited a significant northwards range shift in the North-West Atlantic in recent years (Thorne & Nye 2021). While this trend is less well understood for the North-East Atlantic, genetic monitoring of both MSE and single stranded individuals should continue in future to monitor LFPW response and movement to changing climate conditions by comparing historic samples with more recent samples. Additionally, this would improve our understanding of the impacts of these large MSE deaths, to wider population diversity and adaptive potential.

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Appendix 1: Further Details to Methodology for Genetic Analysis

Genotyping and Fragment Analysis

The output is in the form of an electropherogram, indicating the fluorescence intensities observed for each sample, relative to the size standard. The variation in size of the fragments amplified is due to the differences in the number of microsatellite repeats. In this instance, for these primers the repeat is a size difference of four nucleotides. As LFPW are diploid (i.e. have two copies of each chromosome), a maximum of two alleles (genetic variants) were expected per locus (i.e. a microsatellite marker). The genotype of an individual can be homozygous (two copies of the same allele) or heterozygous (one copy of two different alleles).

Quality Control – Hardy-Weinburgh Equilibrium

HWE predicts that expected genotypes can be predicted based on the observed allele frequencies within a population under the assumptions of random mating, no migration, and no selection; deviations only for a particular locus within a population would indicate that one of the afore mentioned assumptions is out of neutral expectation and that locus should be removed from the analysis.

Relatedness Analysis

Parent-offspring relationships can be determined with higher confidence than other genetic relationships as the offspring must share at least one allele at every locus. In wild populations where sampling will not capture every potential parent, analysis in a statistical framework, such as likelihood, allows the consideration of factors such as error rates and proportion of candidate parents sampled to assess the confidence of assignments (Städele & Vigilant, 2016). The likelihood ratio of a dyad is assessed by testing the putative presence of a relationship dyad (i.e. parent-offspring) given its patterns of allele sharing over the likelihood that the dyad has an alternative relationship (i.e. unrelated). Dyadic relatedness estimators evaluate the amount of genetic material shared by decent between individuals and thus accuracy is dependent on the number of markers typed, their polymorphism and allele frequency distribution. The relatedness coefficient generated should be tested against cutoff values for different levels of kinship which are often expressed as the logarithm of the likelihood ratio (log odds ratios: LOD) to reduce misclassification. Another tool for determining relationships is sibling-ship reconstruction, this is particularly useful when groups are expected to contain full/half siblings, like LFPW. This approach considers the relationships among all genotypes simultaneously. However, polygamous mating in both sexes and the inclusion of unrelated dyad can lower accuracy and result in nonconvergence of results (Städele & Vigilant, 2016).

The potential genetic units identified in COLONY were then re-analysed in ML-R$_{ELATE}$ to test that a second-degree relationship (i.e. half-sibling) was the most likely relationship between all dyads within the genetic cluster. A p-value for the putative relationship (second-degree) was calculated with 10,000 simulations, then the same for the alternative relationship (unrelated). If the absolute value between the two p-values was less than/or equal to 0.025 the putative relationship was retained, and those individuals were designated as a maternal cluster. Maternal clusters are firstly composed of mother-offspring dyads and individuals that have a second-degree relationship with the mother. For second-degree relationship dyads with no mother present among the MSE individuals, if the sibling-ship reconstruction showed

that the half-sibling *r*-value equivalent was based on the same inferred mother and the second-degree relationship was retained after p-value testing, then these individuals were retained in a maternal cluster.

Appendix 2: Sanday, Orkney 2024 Mass Stranding Event

Overview Sanday, Orkney (2024) MSE

On 11 July 2024, a MSE involving 77 LFPW occurred at Tres Ness Beach, Sanday, Orkney (Scotland) (Figure 16). On discovery, 12 individuals remained alive but were euthanized after a full tidal cycle due to their deteriorating condition. Necropsies (n = 36) and tissue sampling (n = 77 + 1 foetus) were conducted post-mortem. Comprehensive sampling, including individual position, was undertaken to inform comparative analyses with the 2023 Isle of Lewis, Tolsta event (Table 11). Genetic and ecological studies are ongoing at multiple UK institutions under SMASS leadership to determine potential causes of this event and assess population health. The genetic analysis of this event was conducted alongside the analysis of the Tolsta event as part of this JNCC project. Stable isotope and molecular foraging ecology analysis has not been completed for these animals and would be a valuable avenue for future work.

Figure 16. Initial discovery of 77 stranded LFPW on Tres Ness Beach, Sanday, Orkney (Photo: E. Neave, 11 July 2024).

Table 11. Summary of the Sanday 2024 MSE, numbers of individuals and samples collected on 11 July 2024.

Globicephala melas	Sanday MSE
Total Animals on discovery	77
Animals Euthanised	18
Animals Refloated	0
Male	36 (20 adults, 14 juveniles, 2 neonates)
Female	41 (27 adults, 14 juveniles, 1 foetus)
Necropsies	36 total (24 females, 12 males)
% Necropsied	47
% Necropsied or Sampled	100
Samples Collected	2,282

Methods

The methodology is the same as described for the Tolsta MSE in Section 4.2 except for the maternal cluster analysis. Due to time constraints, combined analysis with isotope data was not possible.

Kinship Analysis Results

DNA was extracted from 78 tissue samples collected from each individual in the Sanday MSE. Microsatellite amplification was successful for all 12 loci for all 78 individuals.

All samples were analysed for their relatedness to each other. The mean pairwise relatedness was -0.005 (i.e. the majority of pairs of individuals were unrelated) indicating that upon further analysis there will potentially be multiple unrelated pods present within this MSE.

The consensus relationships between individuals from the ML-R$_{ELATE}$, C$_{ERVUS}$ and COLONY results found 31 parent-offspring dyads among the 78 individuals. Including the foetus, there were 20 mother-offspring dyads (adult and juvenile offspring) and three father-offspring (juvenile) dyads. There were seven parent-offspring dyads between adult individuals.

To visualise these connections between individuals, a Fruchterman-Reingold force-directed network was drawn. The relationships in the second-degree cutoff are shown in Figure 17a. In Figure 17b, only the first-degree relationships are retained to show the distinct close relationships and the individuals with no first-degree relationships. An adult male, individual 36, was the least related LFPW present with only one second-degree relationship connecting him with another individual. Interestingly, individual 32, a juvenile male, only had two second-degree relationships with other individuals present within the MSE.

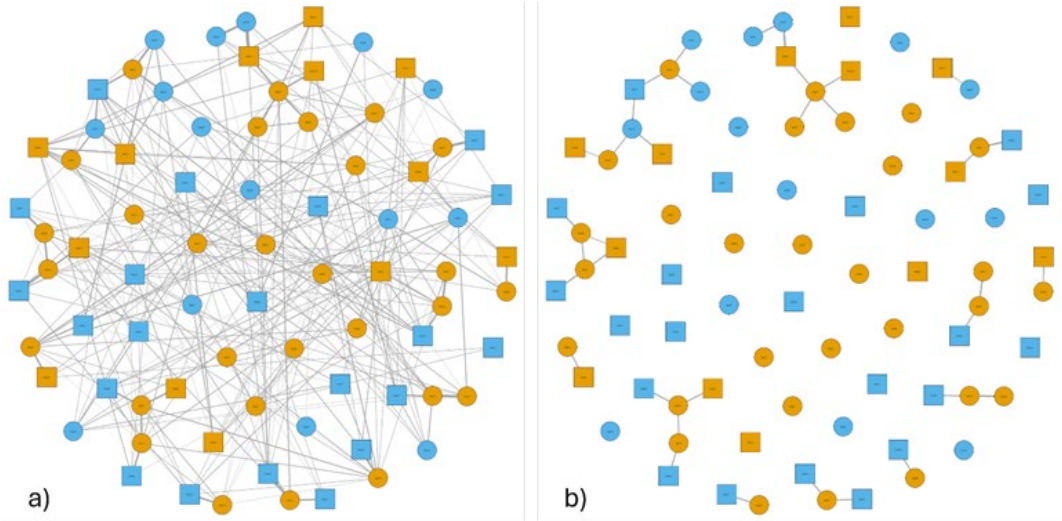

Figure 17. Kinship networks within the Orkney 2024 MSE, adult individuals are represented with a circle, juveniles with a square, males are blue and females yellow in colour. a) shows the second-degree relationships (r = 0.25+, half-siblings & grandparent) and first-degree relationships, the thickness of the connecting lines is indicative of the r-value (i.e. strength of the relationship). b) is a stripped back version of the same network (individuals in the same location) with only the first-degree relationships displayed (r = 0.4+, parent-offspring & full-sibling).

The mother-juvenile dyads were assigned, using the consensus of the three methods and with confidence cutoffs (Table 12). There were 20 mother-juvenile dyads assigned, one of those being the dam-foetus pairing. This means that 43% of the juveniles present on the beach (15 of 35), did not have a mother present. Most notably is the absence of a mother for individual 07, a male neonate with foetal-folds still visible. This neonate would have been born recently and still dependant on his mother. As no adult individuals were refloated during this MSE, this means that these 15 missing mothers did not strand as part of the MSE. This could mean that the mother-offspring bond was separated prior to the MSE beginning or that some individuals present in the at-sea pod complex of LFPW did not strand with the individuals that ended up on the beach. There were no detailed (number of individuals, behaviour, direction of swimming, etc.) reports of LFPW sightings in the area prior to the stranding, although they may have been seen, so the potential degree of temporal separation between stranded offspring and their missing mothers is unknown.

Table 12. The consensus mother – juvenile parings found in the Orkney 2024 MSE. All the juvenile individuals within the MSE are listed, then the assigned mother pair. The juveniles with no mother in the MSE are listed.

Juveniles = 35 + 1 foetus (#F)		Mother-Juvenile pairs	Juveniles NO Mothers
Male = 22	Female = 14		
04	02	01 – 43	02
07	09	08 – 77	07
15	10	13 – 04	09
16	11	33 – 41	11
18	27	33 – 46	15
22	29	35 – 18	16
25	42	35 – 53	25
28	43	37 – 37F	27
30	52	37 – 67	28
31	54	55 – 42	30
32	58	55 – 45	31
41	62	56 – 58	32
45	67	57 – 22	49
46	37F	57 – 54	52
49	-	59 – 64	62
53	-	70 – 52	-
60	-	70 – 60	-
64	-	73 – 65	-
65	-	74 – 68	-
68	-	76 – 71	-
71	-	-	-
77	-	-	-

Spatial analysis within the MSE

Individual location points were used to analyse the proximity of LFPW to their close relatives on the beach. Figure 18 displays the 20 mother-juvenile dyads (from Table 12), positions on the beach relative to the other individuals present in the MSE. The mother-foetus pair is not separated and is circled/labelled within Figure 18. The other mother-juvenile pairs are connected by an arrow pointing to the juvenile, labelled with the pairing IDs.

The neonate with no mother present is displayed with a diamond outline. The other neonate present, 77, did have a mother present, 08, but these two individuals were separated at either end of stranding location (Figure 18).

The mother-juvenile pair 56 – 58 were in close spatial proximity however, not directly next to each other as an unrelated adult female was between them.

Several of the females (33, 35, 55 & 57) had two juvenile offspring present within the MSE. The larger juvenile was further from its mother than the smaller juvenile for three of the four mothers (55's juveniles were the exception however both juveniles were in a similar position on the beach) (Figure 18). This is interesting as the more dependant calf is closer to its mother than its larger sibling is. This implies an element of kinship cohesion however, the mother-offspring dyad is still separate across the stranding location.

Figure 18 shows that mother-juvenile pairings are not consistently found in close proximity to each other, disproving the kinship cohesion hypothesis in the Northern Hemisphere, as well as in the Southern Hemisphere (Oremus *et al.* 2013). If refloating had been possible for this MSE and if juveniles had been refloated with their nearest adult female, assuming close kinship, then this may have contributed to re-stranding as unrelated adults and juveniles would have been refloated together.

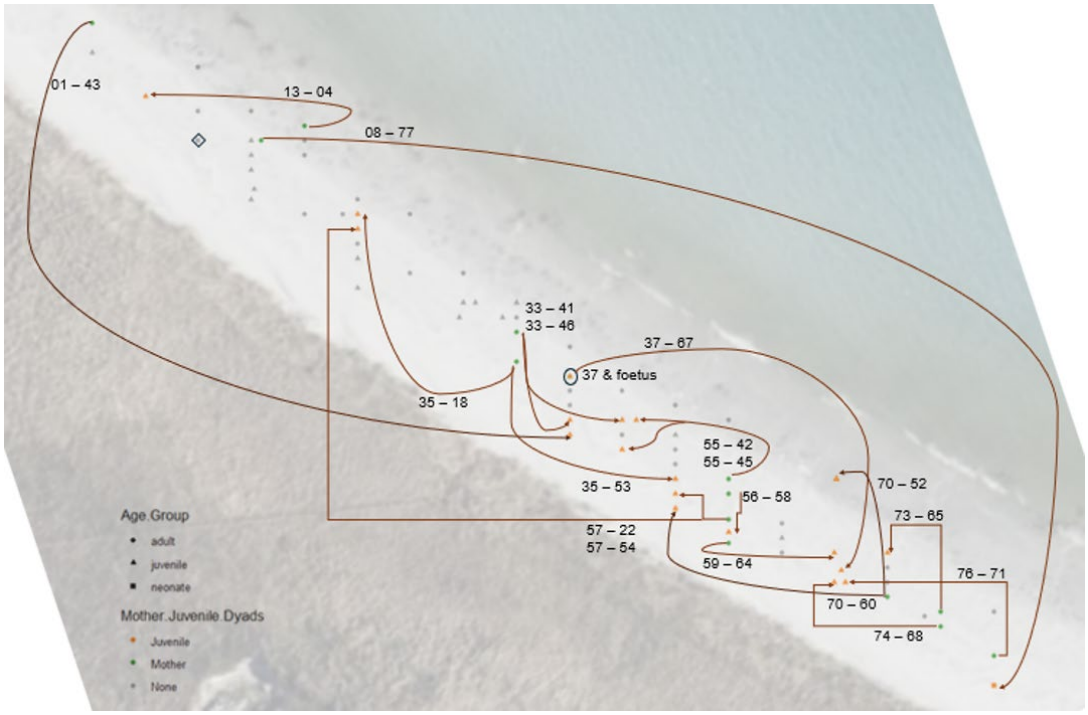

Figure 18. Spatial positions of mother-juvenile dyads on the beach at Sanday. Adults represented by a circle, juveniles by a triangle and neonates by a square. Arrows connect mother and juveniles, with the arrowhead pointing to the juvenile, each arrow is labelled with the individual ID codes. The pregnant female (with a foetus) is circled and labelled within the plot. The neonate with no mother present is outlined with a diamond.

Spatial disruption is evident among all first-degree relationships within the Sanday MSE as seen in Figure 19. Spatial proximity is not expected from father-offspring dyads and not seen in this MSE. Adult female parent-offspring dyads also show no correlation to closeness on the beach, the closest pair (74 – 70) have an unrelated adult between them on the beach (Figure 19). This shows that proximity on the beach should not be considered an indication of close kinship.

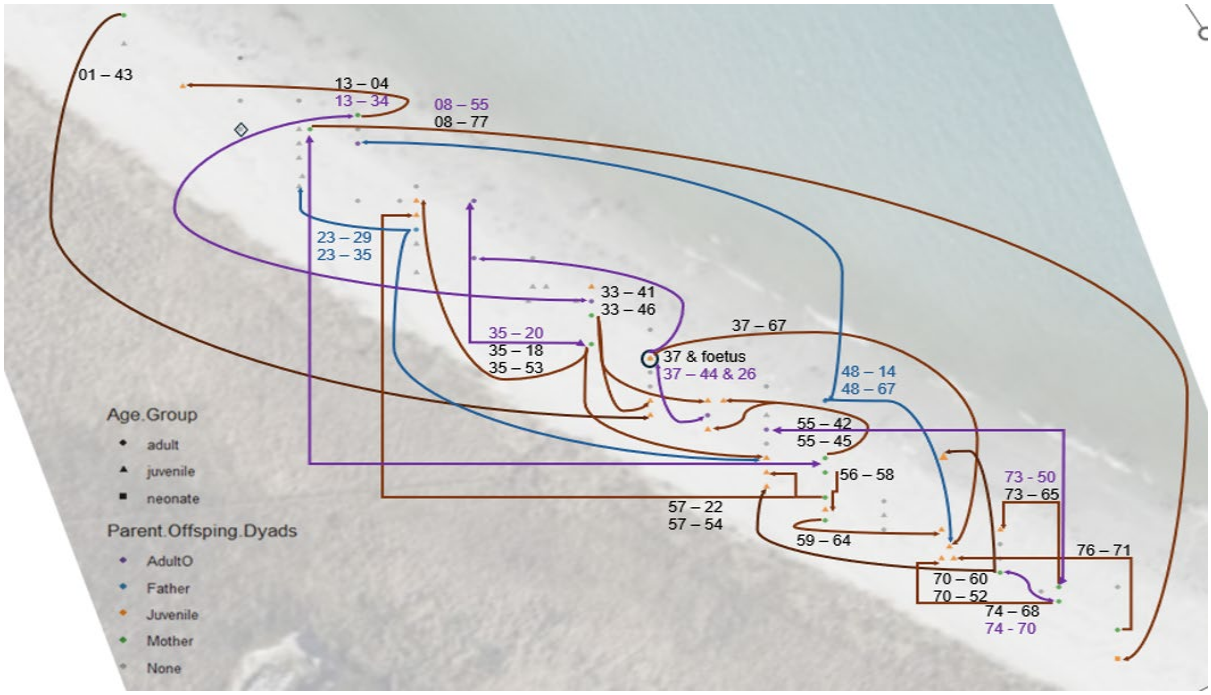

Figure 19. Spatial positions of all Parent-Offspring dyads on the beach at Sanday. Adults represented by a circle, juveniles by a triangle and neonates by a square. Mothers are green, Fathers are blue, Juveniles are orange, Adult-Offspring are purple and indviduals with no first-degree relationships are grey. Brown arrows connect mother and juveniles, with the arrowhead pointing to the juvenile, each arrow is labelled with the individual ID codes. Blue arrows connet father-juvenile dyads and one double-headed arrow connets the adult parent-offspring dyad (48 – 14), ID code labeles are in the colour. The double-headed purple arrows connet female parent-offspring dyads, ID code lables are in the same colour. The pregnant female (with a foetus) is circled and labelled within the plot. The neonate with no mother present is outlined with a diamond.

Conclusions

This MSE contained multiple pod units (families) of LFPW. Despite high relatedness between groups of individuals the overall relatedness of individuals in each event was low. The presence of multiple unrelated pod units and the timing of the month of July, which is within LFPW breeding season, indicates that this large assembly of LFPW were likely in a multigroup association for breeding purposes prior to the MSE (de Stephanis *et al.* 2008). The higher observed heterozygosity (than expected) and presence of private alleles both support that separate pod units were likely in a multigroup breeding association for the largest recorded MSE in Scottish waters.

The Sanday MSE had a high proportion of juveniles with missing mothers (43%). If the stressor instigating the MSE occurred during a babysitting event, that could be an explanation for so many missing mothers. Future work would need to combine behavioural observations with kinship analysis to characterise who is providing alloparental care and at what scale.

Spatial disruption of close kin was evident in the MSE, with juvenile-mother pairs separated across the stranding location. Although some close groupings in space and relatedness were observed, it must not be assumed that spatial proximity is an indication of kinship, as these groupings were in the minority. While large scale refloat efforts were not possible during the Sanday MSE, future MSE rescues should not prioritise refloating juveniles with their nearest adult female neighbour. A better strategy, where possible, would be to refloat as many individuals as possible at the same time and let them re-establish kinship and social bonds away from human influence.