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INTERNATIONAL WHALING COMMISSION

Preliminary report of efficiency and practicability of biopsy sampling, faecal sampling and prey species identification from genetic analyses in 2014, and work-plan for non-lethal research in JARPN II

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ABSTRACT

This paper is the preliminary report of efficiency and practicability of biopsy sampling, faecal sampling and prey species identification from genetic analyses in 2014. It also includes a work-plan for non-lethal research in JARPN II. Biopsy sampling of sei and Bryde's whales has been conducted in many surveys and has been shown to be reasonably efficient. However, the efficiency (the number of obtained samples per targeted individuals) of biopsy sampling was lower than that of lethal sampling. Furthermore, it seems to take too much effort to conduct biopsy sampling for common minke whales as an alternative to lethal sampling in JARPN II. We also have concluded that sampling of faeces from swimming whales is inefficient because of the rare encounter excretion and that sampling of faeces from sea water seems to be highly biased depending on the prey species consumed by whales. The preliminary study of DNA analyses of the content of large intestine of whales using next-generation sequencing (NGS) technologies clearly indicated that the genetic prey ID only from the contents of the large intestine is insufficient to understand feeding habits of the whales, because of their low identification rate of prey species in the large intestine contents. Furthermore, the prey species compositions identified in the large intestine were quite different from those in the stomach. We sampled blubber and prey samples in the 2014 survey, however the analyses of stable isotope and fatty acid could not be conducted because of logistical reason. We are going to analyse these samples and evaluate how these results contribute to the elucidation of the role of whales compared to conventional stomach contents analyses.

KEYWORDS: SCIENTIFIC PERMITS; COMMON MINKE WHALE; BRYDE'S WHALE; SEI WHALE

INTRODUCTION

Some non-lethal techniques have proven practical in Japan's whale research and therefore biopsy sampling has been conducted in Japanese Special Permit surveys through JARPA, JARPA II, JARPN and JARPN II especially in the dedicated sighting surveys. However, we did not evaluate the efficiency of non-lethal techniques such as biopsy sampling, and faeces observation and sampling.

Following the March 31, 2014 Judgment of the International Court of Justice (ICJ) in the case whaling in the Antarctic (Australia v. Japan: New Zealand intervening), the Government of Japan voluntarily reviewed the state of JARPN II. In the process of the review it was decided to evaluate the potential use of the non-lethal techniques.

Here we report the preliminary results of the evaluation of non-lethal sampling from a technical point of view addressing the following points:

(i) whether a tissue and other samples can be obtained by a non-lethal method (*e.g.* biopsy sampling and faeces collection);

(ii) whether enough number of samples can be obtained by the non-lethal method for statistical analysis;

(iii) whether the sample obtained by the non-lethal method can produce scientific information compatible to that produced by lethal sampling; and

(iv) whether the cost for obtaining the sample/producing scientific information is reasonable.

Points (i) and (ii) above are technical, (iii) is analytical, and (iv) is a logistical evaluation.

Experiments related to the above points were carried out in 2014 and will be continued for an additional 2 or 3 years.

The main objectives of JARPN II are to elucidate the feeding ecology of whales and the construction of ecosystem models based on interspecific interactions among whales and their prey species in the western North Pacific. Prey information such as prey species and amount of food taken has been collected for common minke (*Balaenoptera acutorostrata*), sei (*B. borealis*) and Bryde's (*B. edeni*) whales. Therefore the experiments will address whether feeding information derived from non-lethal methods is comparable to that from lethal methods. As potential alternatives to stomach contents analyses from lethal sampling, stable isotope and fatty acid analyses of biopsy samples and genetic analyses of faecal samples have been reported in many marine mammal studies, however we did not conduct these analyses because of a logistical reason.

The ICJ judgement also pointed out that the other similar research program (JARPA II) has not conducted practicability experiments for non-lethal sampling. To respond to this, we first tested the practicability of biopsy sampling and sampling of excreted faeces from swimming whales targeted in JARPN II. Evaluation of whether the samples obtained from biopsy and faecal sampling methods can achieve the objectives of JARPN II which require quantitative information on feeding of baleen whales was made.

This paper reports the progress of non-lethal sampling and preliminary results of the evaluation of non-lethal methods in the 2014 JARPN II with reference to points (i) and (ii) above. Evaluation related to the other points and an overall evaluation will be reported based on the data from JARPN II research for 3 years from 2014.

MATERIALS AND METHODS

The feasibility studies were conducted in three components of JARPN II in 2014, i.e., Offshore component and Coastal components off Sanriku and off Kushiro. Detailed research methods and results are reported in the cruise reports submitted to this meeting (Mogoe *et al*., 2015; Tamura *et al*., 2015; Yoshida *et al*., 2015). Different survey platforms (research vessels) were used for the Offshore and Coastal components. Larger vessels were used in the offshore survey. Equipment used for biopsy sampling was also different for the above noted components as described below.

Sampling methodology (Biopsy, excretion and contents from large intestine)

i) Offshore component

The offshore component of JARPN II was conducted from 16 May to 29 July 2014 using two sighting and sampling vessels (*Yushin Maru* (*YS1*: 724GT) and *Yushin Maru* No.2 (*YS2*: 747GT))(Fig. 1). Biopsy sampling and observation of excretions and sampling faeces were conducted. Contents of stomachs and large intestines were collected from sampled whales on the board of a research-base vessel, the *Nisshin Maru* (*NM*: 8,145GT) in order to study whether faeces can produce comparable information to that from stomach contents.

A dedicated sighting survey, which is independent from the sampling survey, was carried out from 11 May to 29 June 2014, using a research vessel (*Yushin Maru* No.3 (*YS3*: 742GT)). In this sighting survey, only observation of excretions from swimming whales was conducted.

i)-1 Biopsy sampling

Using two sighting and sampling vessels (*YS1* and *YS2*), biopsy sampling was conducted for common minke, sei and Bryde's whales using a compound crossbow (Fig.2).

i)-2 Observation of excretion and faecal sampling

Observation of excretion from the whales and sampling of excreted faeces were conducted for common minke, sei and Bryde's whales. The observed time is defined as duration of approaching to the whale within 0.2 n.mile to end of chasing or observation. If an observer found faeces near surface of the sea water, the faeces were

sampled by circle net with 100μm mesh size (Fig. 4). The sampled faeces were stored at -20°C until analyses. As an experiment for faecal sampling practicability, contents of large intestines obtained from four sei whales and four Bryde's whales were released to the sea, then attempts to collect these were made after one and three minutes using the circle net in order to compare the contents of large intestines and faeces released to the sea.

i)-3 Sampling of large intestine contents of whales sampled

For testing the potential of prey species identification from excreted faeces sampled using genetic analysis, 50g of large intestine contents were collected from 90 sei and 25 Bryde's whales and stored at -20°C for later examination.

ii) Coastal Component off Sanriku

The coastal component off Sanriku of JARPN II was conducted from 26 April to 11 June 2014. Four small type whaling catcher boats were used as the sighting and sampling vessels (*Taisho Maru* No.28 (47.3GT), *Koei Maru* No.8 (32.0GT), *Katsu Maru* No.7 (32.0GT) and *Sumitomo Maru* No.51 (30.0GT))(Fig. 1). Biopsy and faecal sampling experiments were conducted by these vessels. Contents from large intestine were collected from animals sampled in order to study whether faeces can produce comparable information to that from stomach contents.

ii)-1 Biopsy sampling

As a general rule, biopsy sampling was conducted using a compound crossbow after the catch of one common minke whale by each research vessel.

ii)-2 Observation of excretion and faecal sampling

Observation of excretion was conducted for common minke whales targeted for lethal and biopsy sampling. The observed time is defined as duration from the start of chasing for the sampling to hit time of the harpoon or biopsy sampling dart to the animal or to time of abandonment for chasing. In addition, observation of excretion was conducted after the first catch of common minke whales for each research vessel per day as a general rule. If an excretion was observed, sampling of faeces was attempted using the circle net with 100μm mesh. To test the feasibility of sampling faeces for common minke whales at the sea, contents from large intestines collected from three common minke whales were released to the sea water by use of a 100L plastic tub then attempts were made to collect these using the circle net after 15, 30 and 60 seconds.

ii)-3 Sampling of large intestine contents of whales sampled

For testing the potential of prey species identification from excreted faeces sampled using genetic analysis, 100g of large intestine contents from three common minke whales were also collected and stored -20°C for later examination.

iii) Coastal component off Kushiro

The coastal component off Kushiro was conducted from September 4 - 24 with additional sighting survey on September 2 and 3. The four sighting and sampling vessels that conducted the coastal component off Sanriku were used. Biopsy and faecal sampling experiments were conducted by these vessels. Contents from large intestine were collected from animals sampled.

iii)-1 Biopsy sampling

Biopsy sampling was attempted on four days: afternoon of September 2, 3, and 15, and morning of September 19. During that period, biopsy sampling was conducted without lethal sampling, using two compound crossbows, an air gun (LKARTS-Norway), and a Larsen gun (Fig.2).

iii)-2 Observation of excretion and faecal sampling

Observation of excretion was conducted in the same manner as in the Coastal component off Sanriku, for common minke whales targeted for lethal and biopsy sampling. To test the feasibility of sampling of faeces of common minke whales at the sea, contents of large intestines collected from six animals sampled were released to the sea from the deck of vessels, then attempts were made to collect these using the circle net (100μm mesh) after 1 minute.

iii)-3 Sampling of large intestine contents of whales sampled

The experiments were carried out in same manner as for the Coastal component off Sanriku. 100g of large intestine contents were collected from six common minke whales and divided into two halves. One half of each of the samples from the 6 minke whales was stored at -20°C for later genetic analysis. The other half was used for the feasibility test of faeces sampling, as described above.

Analytical methodology

i)-1 Tissue and prey samples for stable isotope and fatty acids analyses

Blubber samples from all whales sampled in both Offshore and Coastal components and main prey species from stomach contents were stored at -20°C. These samples are going to be used for stable isotope and fatty acids analyses. The number of analyses will be decided based on the budget. The results of the analyses will be reported to the 2016 IWC/SC.

i)-2 The DNA analyses of the contents of large intestine by next-generation sequencing (NGS) technologies

50g of the content of the large intestines were collected from the sampled whales and the samples were stored at -20°C until start of DNA extraction. Some of the samples (from ten sei whales, six Bryde's whales, and nine common minke whales) were analysed. Total genomic DNA was extracted from each sample. A set of universal primers for amplifying a part of the COI region of mtDNA was used for PCR amplification (Leray *et al.* 2013; the barcode of life data systems). Amplified PCR products were analysed using an Illumina MiSeq (NGS) to identify the prey species. These results were then compared to those observed from stomach content on an individual base.

RESULTS and DISCUSSION

1. Effort for non-lethal research activity

1-1. Offshore component

For the sighting/sampling vessels, out of 282.9 hours search effort during the survey, total effort that was spent in the sighting survey for non-lethal experiments was 89.6 hours. These efforts were 31.7% of total research time. For sighting vessels, total effort that was spent for the sighting survey of non-lethal experiment was 262.4 hours (Table 1).

1-2. Coastal component of Sanriku

Out of 571.1 hours searching effort during the survey, total effort that was spent in the sighting survey for non-lethal experiments was 60.8 hours. These efforts were expended 10.6% of the total research time (Table 1).

1-3. Coastal component of Kushiro

Out of 309.5 hours of searching effort during the survey, total effort that was spent in the searching for non-lethal experiment was 58.6 hours. These efforts were 18.9% of total research time (Table 1).

2. Biopsy sampling

2-1. Offshore

Thirty-four hours were taken for the biopsy sampling. For sei whales, 33 schools (42 animals) were encountered and targeted for biopsy sampling (Table 2). Of the 63 darts launched for 42 individuals, 21 darts hit and 16 biopsy samples were collected. The average sampling time was 80 minutes per a sample, and the sampling efficiency (the number of obtained samples per targeted individuals) was 0.38. In the case of lethal sampling, the average sampling time was calculated as 27 minutes, and sampling efficiency was 0.87.

For Bryde's whales, 38 schools (39 animals) were encountered and targeted for biopsy sampling (Table 2). Of the 67 darts launched for 39 individuals, 31 darts hit and 25 biopsy samples were collected. The average sampling time was 32 minute per sample and sampling efficiency is 0.64. In case of lethal sampling, the average sampling time was calculated as 13 minutes, and sampling efficiency was 0.93.

There was no chance to conduct biopsy sampling for common minke whales due to few sightings.

2-2. Coastal component off Sanriku

No common minke whales were encountered during the biopsy experiment.

2-3. Coastal component of Kushiro

During the biopsy searching, 11 schools (12 animals) were encountered and 9 animals were targeted for biopsy sampling (Table 2). Of the 14 darts launched for 8 animals chasing for 7.63 hours, five biopsy samples were collected from five individuals. The average sampling time was 92 minutes per sample. The sampling efficiency was 0.56. In case of lethal sampling, it was calculated as 60 minutes for sampling a whale; 32 minutes shorter than for biopsy sampling. If all the 51 samples were collected only with biopsy sampling, time for collecting the samples increased from 51.0 hours to 78.2 hours (around 1.5 times). Biopsy sampling is likely to be affected in relation to larger wind scale, especially for common minke whales. Biopsy dart is smaller and lighter than harpoon for lethal sampling, and smaller body size with no visible blow in this species make it more difficult for chasing the species than for larger baleen whale species, e.g., sei and Bryde's whales. The average time of biopsy sampling in the common minke whale was summarized by Beaufort wind scale (Table 2). Twenty-three minutes was calculated as the time for sampling at the wind scale '1' and 194 minutes at the scale '2'.There was no sampling chance at the scale '0' (calm condition). In case of lethal sampling, it was calculated as 28, 45, 66 and 75 minutes for the scales '0', '1', '2' and '3-4', respectively. From the comparison of the average sampling time for the biopsy and lethal samplings at the same wind scale, it is noted that efficiency of biopsy sampling changed significantly related to the wind scale. The comparison of efficiency between biopsy and lethal sampling methods should be conducted taking the wind scale into account.

3. Observation of excretion and faecal sampling

3-1. Offshore

For sei whales, 679 individuals were observed (Table 3). During the observation effort of 81 hours, excretion was recognized for 21 individuals. Faeces samples were obtained from three individuals using circle net (Fig. 3). The average sampling time was over 25 hours per a sample.

For Bryde's whales, 158 individuals were observed (Table 3). During the observation effort of 38 hours, excretion was recognized for three individuals. However all faeces sampling efforts failed. Because, all faeces dispersed or sink before sampling.

For minke whales, four individuals were observed (Table 3). During the observation effort of 16 minutes, faeces were not recognized.

The re-sampling experiment of contents from the large intestine of sei whales (Fig. 4) showed that the contents derived from whales feeding on copepods tend to float while the faeces from whales feeding on fish tend to sink. This represents serious biases in the analysis of feeding habits by faeces samples.

3-2. Coastal component off Sanriku

On the 49 common minke whales, observation for excretion was conducted for 44.8 hours (Table 3). Throughout the experiment, excretion was not observed and therefore there was no opportunity to collect the faecal samples. In the feasibility test for faeces sampling using contents of the large intestine it was observed that contents from whales feeding on sand lace tend to sink (Fig. 5).

3-3. Coastal component off Kushiro

On the 89 common minke whales, observation of excretion was conducted for 60.6 hours (Table 3). One case of excretion was observed, but a faecal sample could not be collected. It dispersed before sampling. Contents of large intestines from six common minke whales were released to the sea from the deck of sampling vessels (Fig. 6). A part of them could be sampled using net.

4. **Preliminary consideration of efficiency of biopsy and faecal sampling**

This study shows the technical aspects of biopsy and faecal sampling, especially their sampling efficiency. Biopsy sampling from larger whales (sei and Bryde's whales) was shown to be comparatively reasonable regarding sampling efficiency. However, sampling efficiency was lower than that of lethal sampling. We also have reached the conclusion that the sampling of faeces from swimming whales is inefficient because of the rare encounter with excretion and that sampling of faeces from the sea water seems to be highly biased depending on the prey species of the whales. However, faecal sampling experiments will be continued in the 2015 and 2016 surveys to more completely evaluate the potential contribution of non-lethal methods to the research objectives of JARPN II as response to the ICJ judgement and, taking account of recommendations by the Review Panel of JARPA II.

5. DNA analyses of large intestine contents of whales using next-generation sequencing (NGS) technologies

Ten, 6 and 9 large intestine contents samples from sei, Bryde's and common minke whales were provided for next-generation sequencing (NGS) analysis respectively. The preliminary results were shown in Table 4 and Appendix 1.

Success rate of genetic species identification was 40.0% in the sei whale samples, 50.0% in the Bryde's whale samples, 66.7% in the common minke whale samples collected from Sanriku, and 50.0% in the common minke whale samples collected from Kushiro (Table 4). The comparison with the results from the analysis of the stomach contents showed 0% match in the sei whale samples, 33.3% match in the Bryde's whale samples, 50.0% match in the common minke whale Sanriku samples, and 33.3% match in the common minke whale Kushiro samples (Table 4). The comparison of results between stomach contents and large intestine is shown in Appendix 1.

Preliminary findings from data in Table 4 and Appendix 1 are as follows:

1. The prey species compositions identified in the large intestine were quite different from those in the stomach contents.
2. Although this method provides some prey information even from a whale without stomach contents, in some cases, no prey species was identified in the large intestine of the individuals notwithstanding the full stomach contents. In addition, the prey identification rate differed among the whale species.
3. Prey of the prey species were also detected (e.g., Copepoda: *Acartia clausii* in the individual 14NPCS-M019 is known as a major prey of sand lance and not as a prey of common minke whales). This is critical point in feeding ecology study of whales.

The next step to be conducted especially for points 1 and 2 above is to examine whether these results were due to either biological, technical or both reasons. One of the technical reasons which we encountered in this study was that almost all of the PCR products predominantly contained the fragments of the whale sequences, causing low prey species identification rate in the samples. Development of a method which avoids amplifying the whale sequences (*e.g*., Shehzad *et al*., 2012) is now under way, so a better resolution will be obtained in near future. Likewise, a solution should be found to separate amplification of prey of the prey species. This preliminary study clearly indicates that the genetic prey ID only in the large intestine (and faeces) is insufficient to understand feeding habits of the whales.

6. Work plan of Stable isotope and fatty acid analyses

We sampled blubber and prey samples in the 2014 survey, but the analyses of stable isotope and fatty acid could not be conducted because of a budget shortfall, however the samples will be analysed to evaluate how these analyses results contribute the elucidation of the role of whales in the ecosystem compared to the conventional stomach contents analyses.

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Table 1. The research effort time (hours) of each survey.

Item/Area	Offshore SSVs (hours)	Offshore SV (hours)	Sanriku (hours)	Kushiro (hours)
Lethal effort	193.3	-	510.3	250.9
Non-lethal effort	89.6	262.4	60.8	58.6
Rate(%)of non lethal/total	31.7	100.0	10.6	18.9
Total	282.9	262.4	571.1	309.5

Table 2. The results of biopsy sampling

Whale species	Ship	Targeted individuals (A)	Number of shoots (B)	Number of hits (C)	Number of samples (D)	Effort (minute) (E)	Sampling effeciency (sample per trial) (D)/(B)	Average sampling time (minute per sample) (E)/(D)
Sei whale	SSVs	42	63	21	16	1275	0.25	80
Bryde's whale	SSVs	39	67	31	25	789	0.37	32
Common minke whale (Sanriku)	SSVs	0	0	0	0	-	-	-
Common minke whale (Kushiro)	SSVs	9	14	5	5	458	0.36	92 (23)[a] (194)[b]

a : efficiency of sampling at Beaufort wind scale ‘1’ ; b : efficiency at scale ‘2’.

Table 3. The results of faecal sampling

Area	Whale species	Ship type	Number of experiments (inds.)	Observation effort (hours)	Observation of excretion (number)	Faecal sampling (number)	Efficiency of sampling (hours per sample)
Offshore	Sei whale	SSVs	346	75.1	11	3	25.0
		SV	333	5.9	10	0	-
	Bryde's whale	SSVs	116	25.4	1	0	-
		SV	42	12.7	2	0	-
	Common minke whale	SSVs	2	0.1	0	0	-
		SV	2	0.2	0	0	-
Coastal	Common minke whale (Sanriku)	SSVs	49	44.8	0	0	-
	Common minke whale (Kushiro)	SSVs	89	60.6	1	0	-

Table 4. The preliminary results of DNA analyses of large intestine of whales using next-generation sequencing (NGS) analysis

Whale species	Number of analyses (inds.)	Number of prey detected (inds.)	Detection rate (%)	Matching of stomach contents (% of yes)
Sei whale (Offshore)	10	4	40.0	0.0
Bryde's whale (Offshore)	6	3	50.0	33.3
Minke whale (Sanriku)	3	2	66.7	50.0
Minke whale (Kushiro)	6	3	50.0	33.3

Fig. 1. Research vessels used as the sighting and sampling vessel at the offshore component (a) and coastal component (b) of the JARPN II.

Fig. 2. The equipment for biopsy sampling. Compound crossbow (left); air gun (LKARTS-Norway; right upper); Larsen gun (right lower).

Fig. 3. The faecal sampling experiment using circle net at the sea (offshore component).

Fig. 4. The experiment for faecal sampling using contents from large intestine of whales sampled (offshore component). Contents from large intestine (left); contents immediately after throwing into the sea (middle); contents at 1 minute after throwing into the sea and sampling using ring net (right).

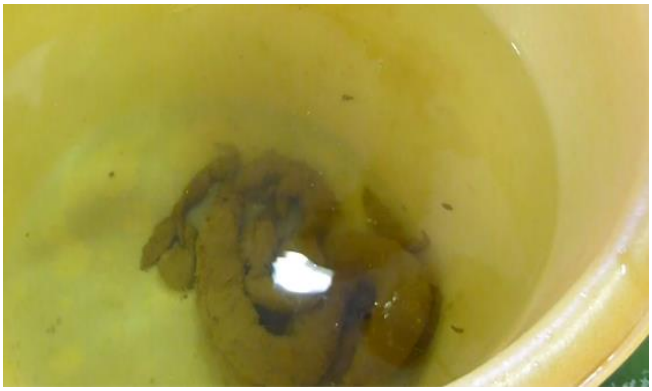

Fig. 5. The experiment for faecal samples using contents from large intestine of whales sampled (coastal component off Sanriku).

Fig. 6. The experiment for faecal samples using contents from large intestine of whales samples, conducted at the deck of a sighting and sampling vessel (coastal component off Kushiro).

Appendix 1. The comparison of results between stomach contents and large intestine.

Species	ID number	Prey species estimating by stomach contents	Prey species estimating by next-generation sequencing (NGS)
Sei	14NPSE001	Mackerels (90%) and Japanese anchovy (10%)	No detected
	14NPSE006	Copepods (99%) and krill (1%)	Krill
	14NPSE018	Mackerels	No detected
	14NPSE044	Japanese sardine (50%), Japanese anchovy (40%) and Mackerels (10%)	No detected
	14NPSE048	Copepods (80%) and Pacific saury (20%)	Krill
	14NPSE052	Copepods	Pacific saury
	14NPSE067	Copepods	No detected
	14NPSE070	Mackerel	No detected
Bryde's	14NPB005	Japanese anchovy	No detected
	14NPB006	Mackerels	Lightfish (*Maurolicus muelleri*)
	14NPB009	Japanese anchovy (90%), Japanese sardine (8%) and mackerels (2%)	Japanese anchovy, Japanese sardine and krill
	14NPB010	Japanese anchovy	No detected
	14NPB016	Japanese anchovy	Krill
	14NPB019	Japanese anchovy (99%) and mackerels (1%)	No detected
Minke	14NPCS-M013	Sand lance	Sand lance
	14NPCS-M019	Sand lance	Copepoda (*Acartia clausii*)
	14NPCS-M021	Sand lance	No detected
	14NPCK-M013	Japanese sardine	No detected
	14NPCK-M015	Japanese sardine	No detected
	14NPCK-M016	Japanese sardine	Japanese sardine
	14NPCK-M017	Walleys pollock and Japanese sardine	No detected
	14NPCK-M019	Japanese sardine	Japanese anchovy
	14NPCK-M027	Walleys pollock and Japanese common sqiod	Japanese common sqiod and krill