

# Detection of food intake in a marine mammal using marine osmolytes and their analogues as dietary biomarkers

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**ABSTRACT:** We report a novel method of investigating foraging in marine mammals based on detecting biomarkers of strictly dietary origin in blood. Arsenobetaine (AsB), the arsenic analogue of the osmolyte glycine betaine, and trimethylamine *N*-oxide (TMAO), an osmolyte used by marine fish and invertebrates, were measured in plasma of lactating Weddell seals *Leptonychotes weddellii* during the postpartum fast and at different stages of lactation. Plasma dietary biomarker concentrations were low in early lactation (<14 d postpartum [d p.p.]; AsB:  $5.5 \pm 2$  ppb As, TMAO:  $66 \pm 20 \mu\text{mol l}^{-1}$ ) and increased 10-fold in late lactation ( $\geq 27$  d p.p.; AsB:  $57 \pm 17$  ppb As, TMAO:  $685 \pm 199 \mu\text{mol l}^{-1}$ ). In lactating females ( $n = 6$ ) monitored longitudinally, plasma TMAO remained low for the first 3 wk p.p. Increases in AsB concentrations observed in late lactation were closely correlated (Pearson correlation  $r = 0.96$ ,  $p < 0.01$ ,  $n = 6$ ) with increases in plasma insulin-like growth factor 1 (IGF1), a growth factor known to reflect nutritional status in other mammals. Two seals outfitted with time–depth recorders began regular deep diving at different times p.p. and showed corresponding differences in biomarker concentrations. Our results indicate that (1) most lactating Weddell seals at the study site (70 % of 20 seals studied) feed after 3 to 4 wk p.p., (2) individual lactating Weddell seals may forage as early as 9 d p.p., and (3) some Weddell seals may consume little or no food throughout lactation. Dietary biomarkers provide a simple method for distinguishing feeding and fasting in free-living marine mammals.

**KEY WORDS:** Foraging · Dietary biomarker · Arsenobetaine · Trimethylamine *N*-oxide · Osmolytes · Lactation · Weddell seal · Marine mammal

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## INTRODUCTION

Large species of marine mammals commonly undergo seasonal fasts associated with migration, moulting, and reproduction (Oftedal 1993, 1997, 2000, Boyd 1998). Among phocid seals that do not enter the water during the lactation period, spatial separation of reproduction and feeding provides a unique opportunity to study the energetics of fasting lactation (Mellish et al.

1999, Mellish & Iverson 2001) as well as hypotheses regarding parental investment (Kretzmann et al. 1993, Wilkinson & van Aarde 2001) and the evolution of capital breeding strategies (Jönsson 1997, Boyd 2000, Crocker et al. 2001). However, in species that feed during lactation (approximately half of all phocid species; Boness & Bowen 1996, Bowen et al. 2001b, Eisert 2003), the relative importance of ‘income’ vs. ‘capital’ (Jönsson 1997) to reproductive energetics is poorly

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understood, because food intake is difficult to detect and quantify in free-living marine mammals.

The general problem of detecting food intake in marine mammals has been addressed by equipping animals with time–depth recorders (TDRs), often in combination with additional instruments. Two-dimensional (Kooyman 1967, Hindell et al. 1991, Bengtson & Stewart 1992, Schreer & Testa 1996) or 3-dimensional (Harcourt et al. 2000, Mitani et al. 2003) TDRs generate dive profiles thought to indicate hunting behaviour, but provide no direct evidence of feeding (Andrews 1998). The combination of TDRs and animal-borne underwater cameras occasionally allows direct observation of foraging, as well as correlation of dive profiles to specific activities (Marshall 1998, Davis et al. 1999, Bowen et al. 2002). Unfortunately, limited storage capacity for images restricts camera observations to hours or a few days at a time, resulting in minimal coverage of biologically relevant periods such as lactation. Jaw movement sensors designed to detect mouth opening by means of electromagnetic contacts glued to the animal's jaws (Bornemann et al. 1992, Plötz et al. 2001) require precise placement of reed contacts and hence prolonged anaesthesia. Jaw opening, although suggestive of feeding, may accompany unsuccessful feeding attempts or occur in behavioural contexts other than feeding. Stomach temperature sensors detect influx of colder exogenous material to the seal's stomach, but this method is susceptible to loss of sensors due to regurgitation, false positives produced when the animal swallows ice or water, and decreasing signal strength with increasing stomach fullness (Bornemann 1994, Hedd et al. 1996, Andrews 1998). All methods that depend on extensive animal-borne instrumentation require animals to be captured prior to a study, possibly affect animal behaviour, and may be limited in scope by cost of instruments or the effort necessary to attach instruments under field conditions.

We propose to apply a novel method of detecting fasting and feeding, the dietary biomarker method, to the study of foraging in marine mammals. The dietary biomarker method is based on the same principle that is used in drug testing: appearance in body fluids of specific compounds—in this case compounds of strictly dietary origin (dietary biomarkers)—represents conclusive evidence of food consumption. Suitable dietary biomarkers should occur in sufficient quantities in the diet, produce a measurable signal in the consumer, and have no sources other than diet. Biomarkers should also undergo minimal metabolism or storage and have simple kinetics to reduce the number of assumptions to be tested. Compounds that meet these criteria have been identified among organic

osmolytes and osmolyte analogues found naturally in marine biota.

Organic osmolytes are small, polar molecules (polyols, amino acids, methylamines and their analogues) that contribute to the osmotic potential of cells or tissues (Baskakov et al. 1998, Lang et al. 1998) and thus protect cells against volume loss in hypertonic media (Fan et al. 1993, Lang et al. 1998) and against freezing damage (Raymond 1994, 1998, Treberg et al. 2002). Glycine betaine (Fig. 1) is a methylamine osmolyte common among plants and animals. Other osmolytes are taxon-specific (Yancey et al. 1982, Carr et al. 1996), for example marine invertebrates and fish use several osmolytes not used by mammals and vice versa (Yancey et al. 1982, Burg 1995). Taxon-specific osmolytes have a key feature that makes them ideal dietary biomarkers: unlike an organism's own osmolytes, which are often under homeorhetic control and retained even in the fasting state, 'foreign' osmolytes absorbed from the diet tend to be rapidly eliminated in urine (Ágústsson & Strøm 1981, Lever et al. 2004) and thus produce a specific, transient signal in the consumer.

Two of several potential osmolyte biomarkers identified by our group are trimethylamine *N*-oxide (TMAO), an organic osmolyte of many marine fish and invertebrates, and arsenobetaine (AsB), an arsenic analogue of the common organic osmolyte glycine betaine (Fig. 1) that is widely distributed in marine biota. To our knowledge, these 2 compounds have not previously been measured in the blood of marine mammals, although AsB has been shown to be the predominant arsenical in tissues of marine amniotes, i.e. marine reptiles, birds, and mammals (Goessler et al. 1998b, Ebisuda et al. 2002, 2003, Kubota et al. 2002, 2003, Fujihara et al. 2003).

The Antarctic Weddell seal *Leptonychotes weddellii* (Phocidae) provides a suitable test case for applying the dietary biomarker method to a free-living marine mammal. The Weddell seal is predicted to fast throughout lactation based on phylogeny (Boness & Bowen 1996), maternal body size (Boness & Bowen 1996, Bowen et al. 2001b), and greater efficiency of energy transfer in fasting lactation (Fedak & Anderson 1982, Costa et al.

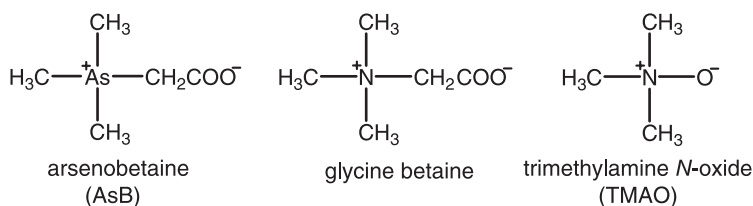


Fig. 1. Chemical structures of arsenobetaine, glycine betaine, and trimethylamine *N*-oxide

1986), yet indirect evidence from dive patterns and body mass changes (Testa et al. 1989, Hindell et al. 2002, Sato et al. 2002) and isolated direct observations (Lindsey 1937, Sato et al. 2002) indicate that some lactating females and/or nursing pups may feed. The aim of our study was to determine onset and prevalence of foraging in lactating Weddell seals and to test the dietary biomarker method in a wild population. Concentrations of AsB and TMAO were measured in lactating Weddell seals during the initial postpartum fast and at various stages during the lactation period. In a subset of seals in late lactation, plasma levels of AsB were compared with plasma levels of insulin-like growth factor 1 (IGF1), a compound known to correlate with re-feeding in other mammals (see 'Discussion'). We also monitored concurrent changes in diving behaviour and plasma biomarker concentrations in 2 lactating females from mid- to late lactation.

## MATERIALS AND METHODS

**Study site and design.** Research was conducted on lactating Weddell seal females between October and December 1998 at the Hutton Cliffs colony, McMurdo Sound, Antarctica. For the purposes of this research, lactation in Weddell seals (42 to 55 d; Lindsey 1937, Kaufmann et al. 1975, Thomas & DeMaster 1983) was considered to consist of 3 stages, early lactation (0 to 13 d postpartum [d p.p.]), mid-lactation (14 to 26 d p.p.) and late lactation ( $\geq 27$  d p.p.). Lactation stage of study animals was ascertained by noting pup birth dates during daily patrols. Individual seals were identified by flipper tags applied by a research group led by Prof. Don Siniff, University of Minnesota, USA.

Adult females were captured by head-bagging (Stirling 1966) and blood samples were collected into heparinised containers by venipuncture from extradural or posterior flipper sites (Cline et al. 1969, Geraci 1971). Blood samples were placed on crushed ice and centrifuged to obtain plasma within 2 h of collection. Plasma was frozen immediately at  $-28^{\circ}\text{C}$  at the field camp and stored frozen until analysis. Three studies were undertaken with animals in this colony: (1) Comparison of plasma biomarker concentrations in early and late lactation: Blood samples were collected from lactating females during early ( $< 14$  d p.p.,  $n = 16$ ) and late lactation (27 to 43 d p.p.,  $n = 20$ ). For this comparison, only 1 sample was used per seal. (2) Change of plasma TMAO in early and mid-lactation: Six lactating females were captured repeatedly at 2 to 6 d intervals. One female was monitored from 4 to 14 d p.p., the remaining 5 females were captured from 2 to 5 until 22 to 27 d p.p. (3) Relation of plasma biomarker concentrations to dive activity: TDRs were used to study dive

behaviour in 2 lactating Weddell seals from 7 and 14 until 38 d p.p. with concurrent monitoring of plasma biomarkers. TDRs (1-channel data-loggers 'pillboxlogger', 8 bit, Driesen & Kern) sampled pressure at 8 s intervals (Bornemann et al. 1998) and were provided by the Alfred-Wegener-Institut für Polar- und Meeresforschung (AWI), Bremerhaven, Germany. TDRs were attached to mats glued to the seals' dorsal pelage as previously described (Bornemann & Plötz 1993, Bornemann et al. 1998). Seals were recaptured by head-bagging at intervals of 4 to 10 d for collection of samples, exchange of data loggers and retrieval of data. Dives exceeding 7 m recorded depth were counted manually after transfer of depth-time data into spreadsheet format (Microsoft Excel).

Experimental procedures involving Weddell seals were approved by the Lincoln University Animal Ethics Committee and conducted under a permit issued by the New Zealand Department of Conservation (28 September 1998) and Antarctica New Zealand Environmental Authorisation no. 98/11. Sample material was imported to the USA for AsB analysis under Marine Mammal Permits 834 and 7631534.

**AsB analysis.** AsB was analysed in Weddell seal plasma in triplicate by graphite furnace-atomic absorption spectrometry (GF-AAS) after extraction and chromatographic separation. The method presented here (Eisert 2003) was developed based on an unpublished method by Trevor Walmsley and Michael Lever (Canterbury Health Laboratories, Christchurch, New Zealand) and is similar in principle to a published procedure by Nixon & Moyer (1992). Plasma samples were extracted with a methanol:water mixture, 9:1 v/v (Goessler et al. 1998a) followed by ion-exchange chromatography to separate quaternary arsonium compounds (AsB) from inorganic and anionic organic forms of arsenic (chemical symbol As) potentially present in samples and also to remove phosphate, which interferes with arsenic determination by GF-AAS (Welz & Sperling 1999). Exclusion of inorganic arsenic ( $\text{H}_3\text{AsO}_{4(\text{aq})}$  at equimolar concentration of AsB) and of phosphate ( $\text{K}_2\text{HPO}_{4(\text{aq})}$ , 800 ppm) was confirmed in preliminary experiments. Plasma extracts (methanol:water) were evaporated to dryness, re-suspended in a small volume of 10 M NaOH, loaded on a disposable strong anion-exchange column (ICH quaternary ammonium, Alltech Associates) and eluted with distilled water. The eluate was concentrated and AsB was determined in the final sample as total arsenic by GF-AAS (Perkin-Elmer 700 graphite furnace atomic spectrophotometer with continuum source background correction, Perkin-Elmer) against AsB calibration standards (purum, Fluka Chemie, CH-9471) using a palladium/magnesium nitrate modifier (Perkin-Elmer; Welz et al. 1988, Deaker & Maher 1999).

Recovery of AsB added to samples before analysis was  $75 \pm 10\%$  from seal plasma ( $n = 4$ ) and  $74 \pm 0.2\%$  from aqueous standards ( $n = 6$ ). Results were corrected for recovery. The characteristic mass was  $15.9 \text{ pg As per } 0.0044 \text{ absorbance units}$ , and the limits of detection (LOD) and quantitation (LOQ) were  $0.06 \text{ ppb As}$  ( $p < 0.01$ , equivalent to  $1.0 \text{ ppb As}$  in undiluted samples) and  $0.2 \text{ ppb As}$  ( $3.5 \text{ ppb As}$  before dilution), respectively. Between- and within-assay coefficients of variation were  $7.6\%$  ( $n = 3$ ) and  $3.8\%$  (mean of 71 samples analysed in triplicate), respectively.

**TMAO analysis.** TMAO was measured in blood plasma in duplicate by high-performance liquid chromatography (HPLC) after extraction and derivatisation by alkylation with 6-methoxy 2-naphthacyl triflate (Happer et al. 2004) using a modification of the method of Lever et al. (1992) as described in Eisert (2003). Derivatised samples were analysed on a Shimadzu Class VP HPLC (Shimadzu) with a RF-10AXL fluorescence detector (Shimadzu, wavelength  $\lambda_{\text{ex}}$  247 nm,  $\lambda_{\text{em}}$  431 nm). Derivatives were separated on a strong cation exchange column (Alltech Adsorbosphere SCX,  $5 \mu\text{m}$ , part no. 287472, Alltech Associates). The mobile phase consisted of propan-2-ol containing  $3.5\%$  water,  $4\%$  dichloromethane (v/v),  $60 \text{ mM}$  triethanolamine and  $120 \text{ mM}$  glycolic acid (w/v) run isocratically at  $1 \text{ ml min}^{-1}$  and  $40^\circ\text{C}$ .

Recovery of TMAO added to plasma samples before analysis was  $97 \pm 2\%$  ( $n = 7$ ). LOD and LOQ were ca.  $15$  and  $25 \mu\text{mol l}^{-1}$  TMAO, respectively. Between- and within-assay coefficients of variation were  $15.6\%$  ( $n = 16$ ) and  $8.2\%$  (mean of 15 samples analysed in quadruplicate), respectively.

Due to logistical constraints, not all samples collected were analysed for both biomarkers.

**Insulin-like growth factor 1 (IGF1) analysis.** IGF1 in blood plasma was measured using an IGF-binding protein (IGFBP)-blocked radioimmunoassay (RIA; Breier et al. 1991, Blum & Breier 1994). Recovery of unlabelled IGF1 added to samples before analysis was  $96 \pm 7.6\%$  ( $n = 14$ ). The  $\text{ED}_{50}$  was  $0.1 \text{ ng per tube}$ , the detection limit was  $0.7 \text{ ng ml}^{-1}$  and the between- and within-assay coefficients of variation were  $10.1$  and  $5.0\%$ , respectively. As degree of crossreactivity between Weddell seal IGF1 and the polyclonal antibody is unknown, IGF1 results represent immunoreactive IGF1 concentrations.

**Calculation of fasting limits of biomarkers.** Weddell seals reportedly remain with their pups for at least the first week after giving birth, providing a minimal estimate for the duration of the postpartum fast (Kaufmann et al. 1975). We assumed that lactating Weddell seals would consume no food during the first week p.p. Plasma concentrations of AsB and TMAO measured at this time were used to estimate 1-tailed  $95\%$  upper confidence limits from the  $t$ -distribution and standard

deviations (Sokal & Rohlf 1969). Plasma biomarker concentrations above these limits (fasting limits) were considered to represent feeding, allowing animals to be classified as feeding or fasting.

**Statistical analysis.** Unless stated otherwise, results are given as mean  $\pm$  SEM. Mean values were compared using Student's  $t$ -test for normally distributed data. For data that were not normally distributed, median values were compared using the Mann-Whitney rank sum test. Linear correlations were evaluated using Pearson rank correlation or Spearman rank correlation as appropriate (Sheskin 2000). Linear regression statistics were computed using the Deming regression procedure (Linnet 1998) with or without logarithmic transformation of raw data.

## RESULTS

### Fasting levels of biomarkers

Plasma concentrations of biomarkers measured in lactating Weddell seals during the first week of lactation ( $\leq 7 \text{ d p.p.}$ ) were (mean  $\pm$  SD)  $5.7 \pm 2 \text{ ppb AsB-As}$ ,  $n = 3$ , and  $45 \pm 20 \mu\text{mol l}^{-1}$  TMAO,  $n = 10$ . Calculated fasting limits were  $11 \text{ ppb AsB-As}$  and  $81 \mu\text{mol l}^{-1}$  TMAO. Seals with plasma concentrations exceeding these limits were considered to be feeding.

### Comparison of plasma biomarker concentrations in early and late lactation

Biomarker concentrations were compared between 2 groups of lactating Weddell seals, early lactation ( $< 14 \text{ d p.p.}$ ) and late lactation ( $\geq 27 \text{ d p.p.}$ ) as shown in Table 1. Mean plasma concentrations of both biomarkers were approximately 10-fold higher in late compared to early lactation (Table 1), and differences in median biomarker plasma concentrations were significant for both AsB (Mann-Whitney rank sum test:  $p = 0.02$ ) and TMAO (Mann-Whitney rank sum test:  $p = 0.03$ ).

In early lactation after the first week p.p. (8 to 13 d p.p.), plasma biomarker concentrations in excess of the fasting limits were observed in 2 out of 5 lactating females, i.e. in 1 of 4 animals for TMAO (Seal M1125,  $321 \mu\text{mol l}^{-1}$  TMAO, 9 d p.p.) and in 1 of 3 animals for AsB (Seal 821,  $13 \text{ ppb AsB-As}$ ,  $30 \mu\text{mol l}^{-1}$  TMAO, 9 d p.p.). Seal M1125 was captured ca. 10 min after returning from a dive but unfortunately, only TMAO was measured. In Seal 821, the combination of elevated AsB and low TMAO plasma concentrations suggests that feeding took place at least a day prior to sample collection. In late lactation, there was good

Table 1. *Leptonychotes weddellii*. Biomarker concentrations and estimated prevalence of feeding by Weddell seals in early and late lactation. Numbers in square brackets indicate median concentrations of arsenobetaine (AsB) and trimethylamine *N*-oxide (TMAO)

	Early lactation	Late lactation
<b>Number of seals studied</b>		
AsB	6	20
TMAO	14	15
<b>Days postpartum</b>		
AsB	7 ± 1	34 ± 1
TMAO	6 ± 1	35 ± 1
<b>Plasma biomarker concentrations</b>		
AsB (ppb As)	5.5 ± 2 [4.8]	57 ± 17 [25]
TMAO (µmol l <sup>-1</sup> )	66 ± 20 [49]	685 ± 199 [380]
<b>Proportion of seals with biomarker concentration &gt; fasting limits</b>		
AsB	17% (1 of 6)	70% (14 of 20)
TMAO	7% (1 of 14)	73% (11 of 15)

agreement between biomarkers with regard to the proportion of lactating Weddell seals sampled that had values exceeding fasting limits, with 14 of 20 of seals (70%) above fasting limits for AsB and 11 of 15 of seals (73%) above fasting limits for TMAO.

Decline of plasma AsB concentrations during early lactation was estimated based on cross-sectional samples below the fasting limit ( $n = 5$ ). Decline of arsenobetaine in early lactation was monoexponential (Fig. 2A inset) and the decay constant was estimated by Deming linear regression analysis of the  $\log_e$  of plasma AsB-As values  $[\ln(y)]$  versus d p.p. ( $x$ ):

$$\ln(y) = -0.33x + 3.39, r = -0.94, p < 0.05$$

In exponential form:

$$y(x) = 30e^{-0.33x}$$

By contrast, there was no significant change in plasma TMAO concentrations during early lactation (Fig. 2B inset).

### Change of plasma TMAO in early and mid-lactation

Plasma TMAO concentrations were monitored longitudinally in 6 lactating females (Fig. 3). Plasma TMAO concentrations remained below the fasting limit for TMAO (81 µmol l<sup>-1</sup>) in all animals during the entire study period, 4 to 14 d p.p. for 1 female and 3 ± 1 to 25 ± 1 d p.p. for the remaining 5 seals. No consistent pattern of change in plasma TMAO concentrations was observed in this group.

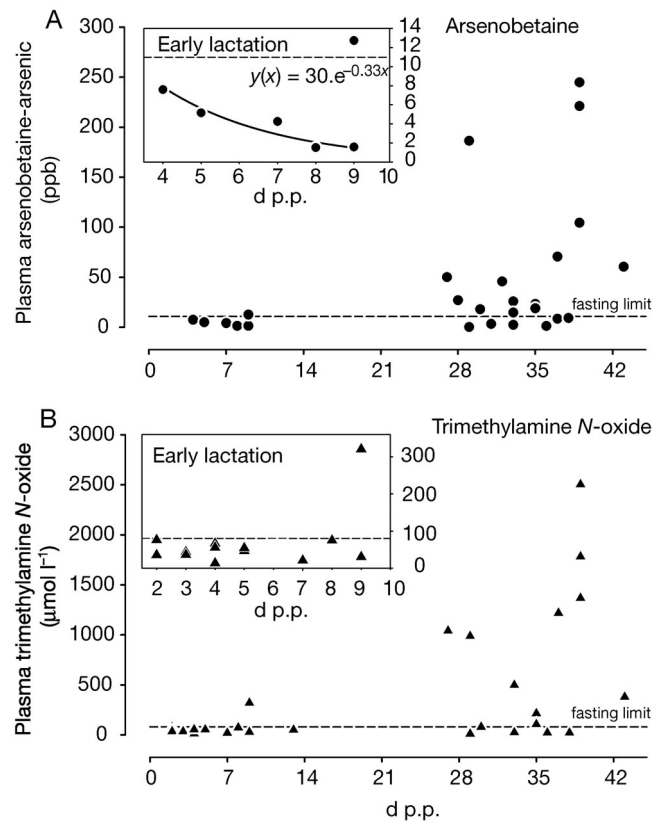


Fig. 2. *Leptonychotes weddellii*. Plasma concentrations of (A) arsenobetaine and (B) trimethylamine *N*-oxide in Weddell seals during early and late lactation. Data from early lactation are shown in expanded form in insets. Exponential decline of arsenobetaine in early lactation was estimated by Deming linear regression analysis after  $\log_e$  transformation ( $r = -0.94$ ,  $p < 0.05$ ). For calculation of fasting limits (---) see 'Materials and methods'

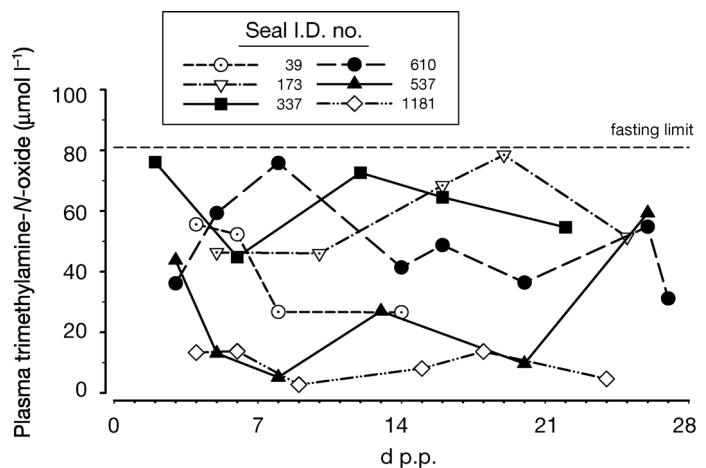


Fig. 3. *Leptonychotes weddellii*. Changes in plasma trimethylamine *N*-oxide concentration in lactating Weddell seals sampled repeatedly during early and mid-lactation. For calculation of the fasting limit (---) see 'Materials and methods'

### Relationship between plasma biomarker concentrations and dive activity

Diving activity and plasma biomarker concentrations were monitored concurrently in 2 lactating females (seals with I.D. tags orange 318 and red 572, referred to as 318 and 572; Fig. 4).

In Seal 318 (Fig. 4A), plasma concentrations of AsB-As declined approximately linearly from 5 ppb at 7 d p.p. to below the LOD (<1 ppb) at 14 d p.p., and remained below 1 ppb until 23 d p.p. Between 23 and 27 d p.p., plasma concentrations of AsB-As increased above the fasting limit, from <1 ppb at 23 d p.p. to 19 ppb at 27 d p.p., and plasma AsB-As was also elevated at 38 d p.p. with 25 ppb. Changes in plasma concentrations of TMAO were similar to those observed for plasma AsB-As, with the difference that TMAO was low (<25  $\mu\text{M}$ ) at the beginning of the study at 7 d p.p. Seal 318 made few, shallow dives

(depth < 50 m) as early as 8 d p.p. (Fig. 4A). Dives exceeded 50 m depth at 14, 21, 23, 26 d p.p. and afterwards on most days until the end of the study period (38 d p.p.). At 28 d p.p., the frequency of all dives and those exceeding 50 m depth increased markedly and remained high until the end of the study period, 38 d p.p. (all dives: 7 to 27 d p.p.,  $3 \pm 1 \text{ d}^{-1}$ ; 28 to 38 d p.p.,  $22 \pm 7 \text{ d}^{-1}$ ). The increase in dive frequency occurred concurrently with the increase in plasma biomarker concentrations (Fig. 4A). Overall median dive depth in Seal 318 was 31 m and maximum depth measured was 304 m.

Seal 572 (Fig. 4B) was studied for a shorter period (14 to 38 d p.p.). Biomarker data and dive records differ noticeably from those of Seal 318. When compared with Seal 318 over the period 14 to 38 d p.p. (Fig. 4), Seal 572 had a greater number of dives per day (median number of dives: Seal 318,  $2 \text{ d}^{-1}$ ; Seal 572,  $16 \text{ d}^{-1}$ ; Mann-Whitney rank sum test,  $p = 0.03$ ) and a

greater proportion of dives exceeded 50 m (dives below 50 m as a percentage of total number of dives per day: Seal 318,  $25 \pm 7\%$ ; Seal 572,  $44 \pm 5\%$ ; Student's  $t$ -test,  $p = 0.04$ ). In Seal 572, concentrations of AsB-As and TMAO were highly variable and elevated above fasting limits throughout the study period (AsB-As, range 12 to 92 ppb, mean  $43 \pm 9 \text{ ppb}$ ; TMAO, range 83 to  $1040 \mu\text{mol l}^{-1}$ , mean  $324 \pm 110 \mu\text{mol l}^{-1}$ ). Increase and decrease of AsB-As and TMAO were approximately synchronous although concentrations of the 2 biomarkers diverged at 21 and 38 d p.p. Observed decreases in biomarker concentrations appeared to be unrelated to preceding changes in dive activity, with the exception of a period of low activity from 25 to 28 d p.p. that was followed by a decrease in plasma concentrations of AsB-As and TMAO. Due to TDR failure, no records exist for the time between 19:00 h 26 d p.p. to 09:00 h 27 d p.p. (Fig. 4B). Overall median dive depth in Seal 572 was 134 m and maximum dive depth measured was 435 m.

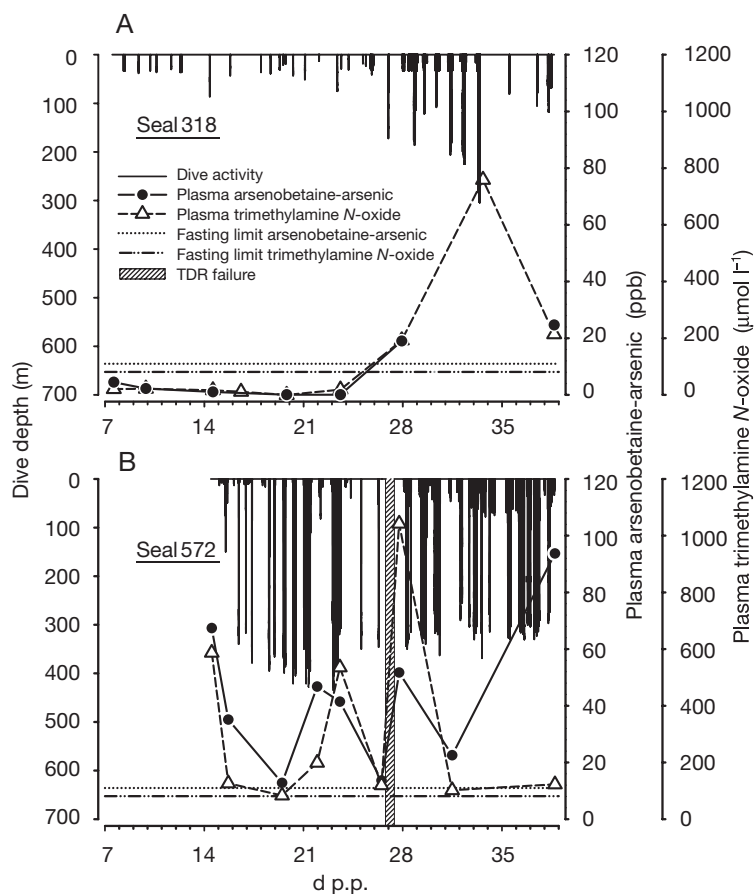


Fig. 4. *Leptonychotes weddellii*. Comparison of dive activity with plasma concentrations of biomarkers in 2 lactating Weddell seals, (A) Seal 318 and (B) Seal 572. Vertical solid lines indicate dive activity as depth below surface. Fasting limits for arsenobetaine-arsenic and trimethylamine *N*-oxide were calculated as described in 'Materials and methods'. Loss of data due to TDR failure in Seal 572 is indicated by cross-hatching

### AsB and IGF1 in late lactation

There was a strong positive linear correlation between plasma concentrations of AsB and IGF1 (Pearson correlation  $r = 0.96$ ,  $p < 0.01$ , Fig. 5) in a subset of Weddell seal females in late lactation ( $n = 6$ , mean  $35 \pm 2 \text{ d p.p.}$ , range 31 to 43 d p.p.). There was no significant relationship between either

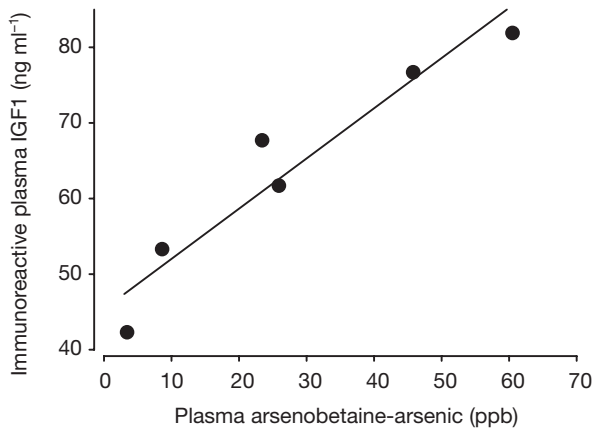


Fig. 5. *Leptonychotes weddellii*. Relationship between plasma concentrations of insulin-like growth factor 1 (IGF1, y-axis) and arsenobetaine (x-axis) in Weddell seals during late lactation, range 31 to 43 d postpartum. By Deming linear regression analysis:  $y(x) = 0.664x + 45.4$ ,  $r^2 = 0.91$ ,  $p < 0.01$ ,  $n = 6$

parameter and time postpartum (Pearson correlation,  $p = 0.26$  [AsB, IGF1]; Spearman rank correlation,  $p = 0.42$  [AsB] and  $p = 0.36$  [IGF1]).

### Correlation of biomarkers AsB and TMAO

There was a strong positive linear relationship between plasma concentrations of AsB and TMAO concentrations after logarithmic transformation (Pearson correlation  $r = 0.88$ ,  $p < 0.0001$ ,  $n = 21$ , including all samples analysed for both biomarkers irrespective of lactation stage). The correlation is marginally improved (Pearson  $r = 0.90$ ,  $p < 0.0001$ ,  $n = 14$ ) if pairs of values below the limits of quantitation for either analyte are excluded (Fig. 6). The latter is consistent with faster elimination of TMAO from the plasma compartment, which would be predicted to cause loss of correlation between AsB and TMAO at low concentrations. This is a possible explanation for the presence of elevated AsB (13 ppb As) and low TMAO ( $30 \mu\text{mol l}^{-1}$ ) concentrations in Seal 821 at 9 d p.p. (see above).

## DISCUSSION

This study presents the use of marine osmolytes and their analogues to detect recent feeding in free-living marine mammals. Applied to the Weddell seal, this dietary biomarker method provides definitive information on the onset and prevalence of foraging in a population of lactating Weddell seals.

### Characteristics of the dietary biomarkers used

The biomarkers used in this study, AsB and TMAO, are common constituents of marine organisms (Yancey et al. 1982, Van Waarde 1988, Cullen & Reimer 1989, Edmonds & Francesconi 1993, Carr et al. 1996) but are not synthesised or stored to a significant degree by mammals (Vahter et al. 1983, Al-Waiz et al. 1987a,b, 1992, Cullen & Reimer 1989, Brown et al. 1990, Mitchell et al. 1997). AsB occurs in marine organisms from zooplankton to teleost fish and is the dominant quaternary arsonium compound, and in most cases the dominant form of arsenic, in almost all marine fish and invertebrates studied (Cullen & Reimer 1989, Shibata et al. 1992, Shibata et al. 1996, Francesconi 2003; Table 2 and references therein). Arsenic compounds found in marine organisms include arsenosugars, arsenolipids, inorganic and anionic organic compounds (arsenate, methylarsonate, dimethylarsonite), and quaternary arsonium compounds other than AsB (trimethylarsonium oxide and the tetramethylarsonium ion). Replacement of AsB as the dominant arsenical by another quaternary arsonium compound, trimethylarsonium oxide, has only been reported for a single species of fish (Edmonds et al. 1997). AsB is retained by marine fish and invertebrates (Cullen & Reimer 1989, Francesconi et al. 1999) but probably in insufficient concentrations to be effective as an osmolyte. Unlike

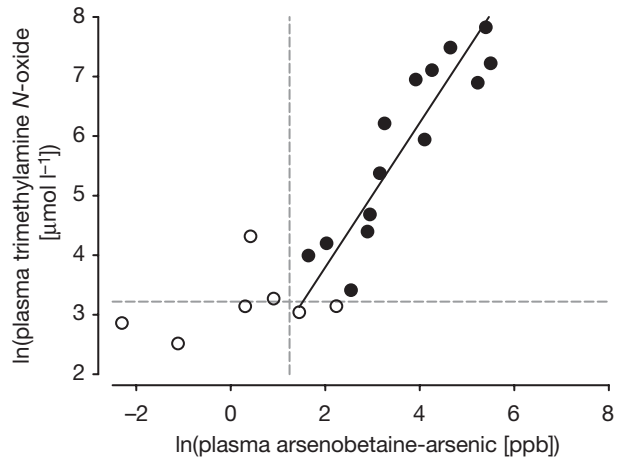


Fig. 6. *Leptonychotes weddellii*. Relationship between plasma concentrations of arsenobetaine (x-axis) and trimethylamine N-oxide (y-axis) in lactating Weddell seals. Limits of quantitation (LOQ) are indicated by dashed lines. (O): values below the LOQ of either method; (●): values above the LOQ. Results of Pearson correlation for all values after  $\log_e$  transformation are  $r = 0.88$ ,  $p < 0.0001$ ,  $n = 21$ . Linear regression of pairs of values above the LOQ of either method (Deming linear regression analysis:  $\ln(y) = 1.21 \ln(x) + 1.37$ ) is shown as a solid line (Pearson correlation  $r = 0.90$ ,  $p < 0.0001$ ,  $n = 14$ )

Table 2. Concentration of total arsenic (As), arsenobetaine (AsB) and quaternary arsonium compounds (QACs) in marine fish and invertebrates. n/r: not recorded; n/m: not measured

Common name	Species	Tissue	Origin	Total As (ppm)	AsB-As (ppm)	AsB (% of total As)	QAC	Source
<b>Cnidarians</b>								
Box jellyfish	<i>Carybdea rastonii</i>	Whole animal	Japan	0.135	n/m	'Major' <sup>a</sup>	n/m	Hanaoka et al. (1999)
<b>Molluscs</b>								
Phoenician neptune	<i>Neptunea lyrata phoenicea</i>	Soft tissue	n/r	3.5	1.1	32	n/m	Cullen & Reimer (1989)
Saul's triton	<i>Charonia sauliae</i>	Muscle	n/r	117	67	58	n/m	Cullen & Reimer (1989)
Roe's abalone	<i>Haliotis roei</i>	Foot muscle	Australia	1	0.9	90	91	Edmonds et al. (1997)
Blue mussel	<i>Mytilus edulis</i>	Soft tissues	W Australia	19.1 <sup>b</sup>	16.6 <sup>b</sup>	87	n/m	Francesconi et al. (1999)
Squid	n/r	Edible parts	Spain	1.6	1.3	56	56	Súñer et al. (2002)
North-Pacific giant octopus	<i>Paroctopus dofleini</i> ( <i>Enteroctopus dofleini</i> )	Muscle	n/r	49	n/m	>90	n/m	Cullen & Reimer (1989)
<b>Crustaceans</b>								
Antarctic krill	<i>Euphausia superba</i>	n/r	n/r	n/m	n/m	60	n/m	Edmonds et al. (1997)
King prawn	<i>Penaeus latisulcatus</i>	Tail muscle	Australia	10–17	9.5–16	95	n/m	Francesconi & Edmonds (1987)
Red crab	<i>Chionoecetes opilio</i>	Soft tissue	Sea of Japan	3.5	3.2	90	n/m	Matsuto et al. (1986)
<b>Cartilaginous fish</b>								
Dogfish (DORM-2) <sup>c</sup>	<i>Squalus acanthias</i>	Muscle	Canada	17.7 <sup>b</sup>	16.0 <sup>b</sup>	90	92	Goessler et al. (1998a)
Star spotted shark	<i>Mustelus manazo</i>	Muscle	n/r	17.3	16.9	98	n/m	Hanaoka et al. (1987)
<b>Teleost fish</b>								
Silver drummer	<i>Kyphosus sydneyanus</i>	Muscle	Australia	1.0	0	0	98 <sup>d</sup>	Edmonds et al. (1997)
Tailor (bluefish)	<i>Pomatomus saltatrix</i>	Muscle	Australia	2.8 <sup>b</sup>	2.6 <sup>b</sup>	94	94	Kirby & Maher (2002)
Tuna (CRM 627) <sup>c</sup>	n/r	Muscle	n/r	4.8 <sup>b</sup>	4.1 <sup>b</sup>	85	85	Nakazato et al. (2000)
European plaice <sup>c</sup>	<i>Pleuronectes platessa</i>	Muscle	North Sea	43.2 <sup>b</sup>	40.3 <sup>b</sup>	95	95	Larsen et al. (1997), Súñer et al. (2000)
European anchovy	<i>Engraulis encrasicolus</i>	Edible parts	Spain	3.2	2.7	82	85	Súñer et al. (2002)
European pilchard	<i>Sardina pilchardus</i>	Edible parts	Spain	4.3	3.4	70	70	Súñer et al. (2002)
European hake	<i>Merluccius merluccius</i>	Edible parts	Spain	2.0	1.8	85	86	Súñer et al. (2002)
Megrim	<i>Lepidorhombus sp.</i>	Edible parts	Spain	3.3	2.8	85	85	Súñer et al. (2002)

<sup>a</sup>AsB accounted for most of the arsenic present but was not quantified; concentrations are on a wet weight basis unless indicated:  
<sup>b</sup>on dry matter basis; <sup>c</sup>standard reference material; <sup>d</sup>95% trimethylarsonium oxide, 3% tetramethylarsonium

other forms of arsenic, AsB has very low toxicity (Cannon et al. 1983, Shibata et al. 1992, Sakurai & Fujiwara 2001), presumably because vertebrates are incapable of breaking down AsB (Vahter et al. 1983, Edmonds & Francesconi 1988, Cullen & Reimer 1989, Yoshida et al. 1998). Despite the structural similarity between AsB and glycine betaine (Fig. 1), AsB is not metabolised by the mammalian enzyme betaine-homocysteine transferase (BHMT) that demethylates glycine betaine (Lee et al. 2004).

TMAO is an important osmolyte in marine fish and invertebrates and is typically present in tissues in remarkably high concentrations of 0.2 to 1.5% by wet mass (Yancey & Somero 1980, Yancey & Siebenaller 1999; Table 3). In addition to its role in osmoregulation, TMAO is thought to stabilise enzymes of deep-sea animals against pressure-induced denaturation (Kelly & Yancey 1999, Yancey & Siebenaller 1999), contribute to buoyancy in cartilaginous fish (Withers et al. 1994) and act as an antifreeze in teleosts (Raymond 1994, 1998, Raymond & de Vries 1998, Treberg et al. 2002).

In mammals, TMAO and AsB ingested with food are absorbed rapidly and completely (Vahter et al. 1983, Al-Waiz et al. 1987b) and are excreted unchanged via urine (Vahter et al. 1983, Al-Waiz et al. 1987b, Brown et al. 1990, Svensson et al. 1994, Vahter 1994, Mitchell et al. 1997, Yoshida et al. 1998), with no demonstrable retention of TMAO in body tissues (Norris & Benoit 1945, Smith et al. 1994). TMAO ingested with food is absorbed as trimethylamine from the intestinal tract after reduction by gut bacteria and re-oxidised by the liver to TMAO (Al-Waiz et al. 1987a). Low levels of TMAO present in body fluids in the absence of seafood consumption (Smith et al. 1994, Svensson et al. 1994, Lundh et al. 1995) are likely derived from breakdown of other trimethyl compounds (e.g. choline, carnitine and glycine betaine) by gut bacteria (Holmes et al. 1997). Marine foods are apparently the only significant source of elevated concentrations of TMAO in plasma and urine unless alternative precursors are administered in pharmacologic doses (Zeisel et al. 1989, Zhang et al. 1999). Following oral administration of trimethylamine in rats, plasma TMAO concentrations peaked at



ca. 1 h and decreased monoexponentially with an approximate half-life of 2 to 3 h (Smith et al. 1994, Nnane & Damani 2001). In studies with radioactively labelled TMAO, 95 % of an oral dose was excreted from the body within 24 h in rats (Mitchell et al. 1997) and humans (Al-Waiz et al. 1987b). In humans, the maximum rate of excretion of TMAO occurs at 3 to 6 h after administration (Al-Waiz et al. 1987b).

AsB is less rapidly excreted than TMAO. A fraction of plasma AsB is thought to enter cells due to its structural similarity to glycine betaine (Vahter et al. 1983, Randall et al. 1996, Fujihara et al. 2003), and this sequestration decreases the rate of elimination of ingested AsB. However, unlike inorganic arsenic that binds to plasma and tissue proteins (Zhang et al. 1998, Benramdane et al. 1999, Hindmarsh 2000), exchange of AsB with intracellular pools of glycine betaine is reversible and AsB is progressively eliminated from the body. Most of a dose of  $^{73}\text{As}$ -labelled AsB administered to rabbits (75 %) and to rats and mice (99 %) was eliminated from the body in 3 d (Vahter et al. 1983). In humans, less than 1 % of a dose of AsB was retained in the body after 24 d (Brown et al. 1990). Following fish consumption, plasma AsB concentrations in humans peaked at ca. 2 h after the meal and declined in 2

distinct phases with respective half-life values of ca. 7 to 12 h and ca. 63 h (Lehmann et al. 2001).

In amniotes, AsB and TMAO are of dietary origin and elevated levels of these markers in body fluids indicate recent ingestion of food of marine origin. Regular intake of marine fish and invertebrates results in high concentrations of AsB in tissues (Ebisuda et al. 2002, Kubota et al. 2002, 2003, Fujihara et al. 2003) and of both AsB and TMAO in plasma and urine (Mohri et al. 1990, Svensson et al. 1994, Lehmann et al. 2001). In the postabsorptive state, AsB and TMAO are gradually eliminated from the body, with TMAO falling to a low baseline within 1 to 2 d and AsB disappearing completely within days to weeks unless replenished by dietary intake.

### Application of the biomarker method to Weddell seals

Weddell seals were previously thought to consume little or no food during lactation (Ofstedal et al. 1987, Tedman & Green 1987). More recently, it has been suggested that lactating Weddell seals commence feeding after an initial postpartum fast (Testa et al.

Table 3. Concentration of trimethylamine *N*-oxide (TMAO) in marine fish and invertebrates. n/r: not recorded

Common name	Species	Tissue	Origin	TMAO (mmol kg <sup>-1</sup> ) <sup>a,b</sup> (%) <sup>c</sup>		Source
<b>Crustaceans</b>						
Antarctic krill	<i>Euphausia superba</i>	Muscle	n/r	30	0.22	Carr et al. (1996)
Dock shrimp	<i>Pandalus danae</i>	Muscle	North Pacific	76	0.57	Kelly & Yancey (1999)
Blue crab	<i>Callinectes sapidus</i>	Muscle	Florida	20	0.15	Carr et al. (1996)
Tanner crab	<i>Chionoecetes bairdi</i>	Muscle	North Pacific	22	0.17	Kelly & Yancey (1999)
<b>Molluscs</b>						
Antarctic pteropod	<i>Clione antarctica</i>	n/r	Antarctica	112	0.84	Seibel & Walsh (2002)
American oyster	<i>Ostrea virginica</i> ( <i>Crassostrea virginica</i> )	Mantle fluid	n/r	240	1.8	Hatton & Gibb (1999)
Pacific razor clam	<i>Siliqua patula</i>	Soft tissue	n/r	15	0.11	Carr et al. (1996)
Market squid	<i>Loligo opalescens</i>	Mantle	North Pacific	48	0.36	Kelly & Yancey (1999)
Argentine shortfin squid	<i>Illex argentinus</i>	Mantle	n/r	55	0.41	Carr et al. (1996)
Common octopus	<i>Octopus vulgaris</i>	Mantle	n/r	8.5	0.06	Seibel & Walsh (2002)
<b>Cartilaginous fish</b>						
Greenland shark	<i>Somniosus microcephalus</i>	Frozen flesh	n/r	140–173	1.1–1.3	Anthoni et al. (1991)
Shovelnosed ray	<i>Aptychotre mata vincentiana</i>	Muscle	Australia	162	1.2	Withers et al. (1994)
<b>Teleost fish</b>						
Saffron cod	<i>Eleginus gracilis</i>	Muscle	Alaska	76	0.57	Raymond (1998)
Pacific cod	<i>Gadus macrocephalus</i>	Muscle	North Pacific	46	0.35	Kelly & Yancey (1999)
Ocean pout	<i>Macrozoarces americanus</i>	Muscle	Canada	38	0.28	Raymond (1998)
Pacific herring	<i>Clupea harengus pallasii</i>	Muscle	Alaska	48	0.36	Raymond (1998)
Starry flounder	<i>Platichthys stellatus</i>	Muscle	Alaska	27	0.20	Raymond (1998)
Antarctic toothfish	<i>Dissostichus mawsoni</i>	Muscle	McMurdo Sound	154	1.2	Raymond & de Vries (1998)
Antarctic dragonfish	<i>Gymnodraco acuticeps</i>	Muscle	McMurdo Sound	148	1.1	Raymond & de Vries (1998)

<sup>a</sup>Units are mmol l<sup>-1</sup> for liquids  
<sup>b</sup>For comparison, the concentration of TMAO in seawater is 3.2 to 64 nmol l<sup>-1</sup> (Hatton & Gibb 1999)  
<sup>c</sup>Mass % by wet weight

1989, Hindell et al. 2002, Sato et al. 2002), but the evidence for feeding has been mostly indirect, such as changes in dive activity (Testa et al. 1989, Hindell et al. 1999, Sato et al. 2002) and lowered rates of maternal mass loss both in absolute terms (Hill 1987) and relative to pup mass gain (Testa et al. 1989). Incidents of feeding by lactating Weddell seals have been recorded with animal-borne cameras (Sato et al. 2002), but observations are too few (<2% of recorded images in Sato et al. 2002 showed actions consistent with food intake) to assess the prevalence of feeding in lactating Weddell seals.

Presently, the application of the biomarker method relies on the assumption that the kinetics of uptake and excretion of AsB and TMAO in Weddell seals are similar to those observed in other mammals (see above). Changes in plasma biomarker concentrations observed during the first week p.p., when Weddell seals remain with their pups and fast (Kaufmann et al. 1975), support this assumption. Between 4 and 9 d p.p., plasma AsB concentrations declined exponentially (Fig. 2A inset) as AsB ingested prior to parturition was gradually eliminated. A tentative estimate of the half-life of AsB in Weddell seals based on these cross-sectional data is ca.  $50 \pm 7$  h, close to the estimate of 63 h in humans (Lehmann et al. 2001). At this rate of decline, plasma AsB would be expected to fall below the detection limit of 1 ppb at about 1 to 2 wk p.p., as seen in Seal 318 (Fig. 4A). In contrast to AsB, there was no consistent decrease in plasma TMAO concentrations with time between 2 and 7 d p.p., either in cross-sectional data (Fig. 2B inset) or in seals sampled repeatedly (Fig. 3). The most likely explanation is that due to the presumably shorter half-life of TMAO in Weddell seals, plasma concentrations of this biomarker had decayed to a physiological baseline by the beginning of sample collection at 2 d p.p.

Based on a limited number of samples ( $n = 3$  for AsB and  $n = 10$  for TMAO), biomarker concentrations during the first week p.p. were used to calculate 95% confidence limits for maximal plasma concentrations of AsB and TMAO consistent with fasting. The fasting limit for AsB calculated from samples collected during the first week of lactation represents a conservative estimate, because plasma AsB concentrations may be expected to continue to decline in animals that continue to fast, for example in Seal 318 (Fig. 4A). It is therefore unlikely that animals were erroneously classified as feeding, although it is possible that feeding animals were mis-classified as fasting in mid- and late lactation as a result of setting the fasting limit for plasma AsB too high. The fasting limit for TMAO is well supported, given the greater number of samples and lack of change in plasma TMAO concentrations during the first week p.p. More rapid elimination of

TMAO means that seals would be erroneously classified as fasting if sample collection took place too long after food consumption and animals fed infrequently. However, since there was good agreement between the proportion of seals classified as feeding during late lactation using either AsB or TMAO values (Table 1), this potential error appears to have been minimal.

Classification of lactating Weddell seals as feeding or fasting using the biomarker method produced 3 main results with regard to the prevalence and onset of feeding during lactation in this species:

Firstly, markedly (ca. 10-fold) higher mean plasma biomarker concentrations in late lactation compared with early lactation indicate that Weddell seals commence foraging during the lactation period. Given the estimated biological half-life values of AsB and TMAO, increases in plasma biomarker concentrations at 3 wk p.p. and later can only derive from recent food intake and not from food consumed prior to parturition. Also, good agreement between estimates of feeding based on either AsB or TMAO in late lactation, and a close correlation between AsB and TMAO concentrations in plasma (Pearson correlation:  $r = 0.88$ ,  $p < 0.001$ ; Fig. 6) support the proposition that food intake typically took place shortly before sample collection. If feeding was infrequent, faster elimination of TMAO relative to AsB should result in greater discrepancy between the 2 biomarkers than was observed (note the discrepancy in biomarker concentration in Seal 821 at 9 d p.p. and in Seal 572 at 38 d p.p. [Fig. 4B]).

Secondly, both longitudinal and cross-sectional samples indicate that the initial fast lasts for 3 to 4 wk in most but not all lactating Weddell seals studied. With the exception of 1 animal (Seal 572), females monitored for at least the first 3 wk p.p. ( $n = 6$ ) fasted during this period. One female was only studied until 14 d p.p. and fasted during this time (Fig. 3). Seal 318 fasted at least until 23 d p.p. and appeared to forage for the remainder of the study. Seal 572 foraged throughout the study period from 14 to 38 d p.p., or more than 50% of the estimated lactation period in this species. Between 8 and 14 d p.p., 2 of 5 lactating females sampled were suspected to be feeding at 9 d p.p. (Fig. 2). After 4 wk p.p., approximately 70% of the sample population had fed recently.

Thirdly, the interpretation of elevated plasma AsB concentrations as indicative of re-feeding in animals during late lactation is supported by comparison with plasma concentrations of IGF1 (Fig. 5). Circulating IGF1 concentrations fall in response to a reduction in intake of protein or energy or both in rats, cats, dogs, domestic ruminants, humans, and northern elephant seal pups, and are restored by refeeding (Eigenmann et al. 1985, Thissen et al. 1994, Maxwell et al. 1998, 1999, Breier 1999, Filho et al. 1999, Frystyk et al. 1999,

Friedl et al. 2000, Ortiz et al. 2003). Hence if elevated AsB concentrations in Weddell seals during late lactation signalled re-feeding, then a positive correlation should exist between circulating levels of AsB and IGF1. Although the comparison could be made only for a small number of lactating females ( $n = 6$ ), there was a strong positive correlation between plasma concentrations of AsB and IGF1 (Pearson correlation,  $r = 0.96$ ,  $p < 0.01$ ).

Taken together, these findings suggest that (1) a large proportion of lactating Weddell seals in the study population commence foraging after 3 to 4 wk p.p., (2) individual Weddell seals may forage as early as 9 d p.p., and (3) it is possible that some Weddell seals may consume little or no food during lactation. The last point is suggested by the observation that ca. 30% of females sampled after 27 d p.p. and 30 to 40% of females sampled after 35 d p.p. had biomarker levels below the fasting limits.

#### Comparison with dive records

Results of simultaneous monitoring of dive activity and biomarker levels in 2 lactating Weddell seals revealed that although dive activity corresponded with food intake in general, diving per se did not equate to food intake. For example, Seal 318 (Fig. 4A) commenced diving at 8 d p.p. but did not start foraging until 23 to 26 d p.p. Although criteria for distinguishing feeding dives from other dives have been proposed (Kooyman 1967, Schreer & Testa 1996, Hindell et al. 2002), these criteria in general lack independent confirmation (Andrews 1998) or may be contradicted by direct evidence from underwater cameras (Davis et al. 1999), e.g. the assumption that Weddell seals feed only at depths greater than 50 m (Sato et al. 2002). In Seal 572 (Fig. 4B), periods of intensive diving were followed by both increases and decreases in observed biomarker levels, suggesting that not all foraging attempts were successful. The divergence of plasma concentrations of AsB and TMAO at 22 and 38 d with relatively low TMAO levels is probably due to the faster elimination of TMAO and suggests that little or no food was caught during the day preceding sample collection. Further concurrent studies of biomarkers and dive activity are required to elucidate the relationships of food intake and dive depth, dive profiles, and dive frequency.

#### Biological relevance of findings

A large percentage of the Weddell seals we studied fed during lactation, in agreement with theoretical

considerations of energy and substrate requirements of lactating females that suggest that an unknown but possibly significant proportion of breeding females has insufficient body stores to complete lactation without feeding (Eisert 2003). However, the close correlation between maternal mass loss and pup mass gain observed by Tedman & Green (1987) in Weddell seal females of large initial size suggests ingestion of little or no food during lactation (Tedman & Green 1987, Testa et al. 1989, Bowen et al. 2001a). Our observation that ca. 30% of lactating Weddell seals sampled during the latter half of lactation appeared to be fasting is consistent with the possible co-existence of capital (fasting) and mixed income-capital (foraging) lactation strategies within the study population. Capital breeding strategies, of which fasting lactation is an extreme example, are considered advantageous when the availability of food during the critical period is poor or unpredictable, whereas income breeding strategies are favoured when there is dependable access to food (Jönsson 1997). Results in lactating harbour seals *Phoca vitulina*, a species that forages during lactation, indicate that feeding does not fully compensate for smaller body stores in lighter females and that pup mass gain and pup weaning mass depend primarily on initial maternal body mass despite foraging by lactating females (Bowen et al. 2001b). There are currently insufficient data to assess to what extent lactating Weddell seals depend on supplementary feeding to complete lactation. A strong dependency, in some or all females, on local food resources for successful lactation might limit breeding colonies to areas of local prey abundance, or result in vulnerability of populations to annual or long-term changes in prey availability, as might occur due to changes in sea ice or shifts in water currents.

#### Limitations of the dietary biomarker method

Circulating biomarker levels depend primarily on 4 factors: (1) concentrations in prey, (2) amount and type of prey ingested, (3) kinetics of uptake and elimination and (4) time of sampling relative to time of food consumption. It is therefore possible to fail to detect food intake with the biomarker method if the food consumed contains low or zero biomarkers, if the amount consumed was small, or if samples are collected too soon (before absorption) or too late (after elimination) relative to the feeding event. Fortunately, both AsB and TMAO are virtually ubiquitous in marine biota, and using several biomarkers reduces the risk of failing to detect food consumption. Further work is required to extend our knowledge of biomarker concentrations in prey, and possible sources of variability

(ontogeny, season, reproductive status, habitat, etc.). It is also essential to quantify kinetics of uptake and elimination of dietary biomarkers in pinnipeds and other marine mammals, and how kinetics may be affected by meal size, activity (e.g. diving vs. resting), species, body size, and other factors.

Lack of data on the kinetics and the dose-response relationship of dietary biomarkers in marine mammals currently precludes accurate estimation of the minimum food intake detectable with the biomarker method. However, assuming that AsB distributes into total body water, we estimate that consumption of 1 kg of prey containing 1 ppm AsB-As (Table 2) should be detectable for at least 24 h after feeding in a 400 kg Weddell seal. For comparison, fish consumption in the adult Weddell seal may exceed 50 kg in a day (Caelhaem & Christoffel 1969). TMAO is present in such high concentrations in marine biota (Table 3) that food consumption by a fasting animal is likely to be detected for even small intakes as long as sample collection takes place within 12 h after feeding. Hopefully, these estimates can be refined as data on biomarker kinetics in marine mammals become available.

The simplified method of analysis for AsB presented here measures quaternary arsonium compounds (QACs) and thus may include compounds other than AsB. However, QACs other than AsB are also strictly dietary and are rarely present in more than trace amounts, with AsB accounting for >95% of QACs in almost all marine animals studied (see Table 2 and references therein). The analytical method presented in this study requires relatively simple sample preparation and only moderately expensive instrumentation (GF-AAS) and may therefore be of greater practical use than more complex methods that measure individual QACs (e.g. Ackley et al. 1999, Ebdon et al. 1999, Gómez-Ariza et al. 2000).

#### Potential applications of the dietary biomarker method

Because the dietary biomarker method only requires collection of plasma samples, it is well suited to test the effect of individual variables, such as body mass, on foraging activity in species with income or mixed income/capital lactation strategies, whether in cross-sectional studies or in longitudinal studies of individual animals over long periods of time. The biomarker method can also provide an independent confirmation of food intake if used together with other methods of estimating foraging activity, such as isotopic methods (Bowen et al. 2001b) or 2- and 3-dimensional TDRs (Schreer & Testa 1996, Hindell et al. 2002, Davis et al. 2003).

AsB is the single most abundant arsenical in liver, muscle and kidney in all predatory marine amniotes studied, including seals, cetaceans, sea birds, and sea turtles (Edmonds et al. 1994, Ebisuda et al. 2002, Kubota et al. 2002, 2003). Thus AsB may be a useful biomarker for species feeding on a wide range of prey including fish and cephalopods (e.g. odontocetes, phocid seals), benthic invertebrates (e.g. walrus, bearded seal), pelagic crustaceans (e.g. fur seals, penguins), zooplankton (baleen whales) and cnidarians (e.g. sea turtles). AsB distributes in soft tissues including adipose tissue (Vahter et al. 1983, Brown et al. 1990, Ebisuda et al. 2003), suggesting that this biomarker could potentially be measured in tissue samples instead of blood plasma. Thus it may be possible to apply the biomarker method to marine amniotes from which dart biopsies but not blood samples can be collected, such as the large whales.

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