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Department of Arctic and Marine Biology

Sampling Site and Potential Errors in Estimating Total Body Water and Water Turnover Rate in Fasting Hooded Seals (*Cystophora cristata*)

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Master thesis in Biology BIO-3950

May, 2014



FACULTY OF BIOSCIENCES, FISHERIES AND ECONOMICS
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Abstract

The purpose of this project is to study in detail the water balance and methodological aspects of the use of the tritiated water method of hooded seals (*Cystophora cristata*). Following 24 hours of fasting, 5 sub-adult hooded seals aged 1.5 years were injected intravenously with a bolus dose of tritiated water through a catheter inserted into the extradural intravertebral vein (EDV) at the level of the lumbar vertebra. A second catheter was inserted at a lower level of the EDV and blood samples collected from both at different intervals. An additional blood sample was collected from the femoral vein. The seals were then reintroduced into seawater and fasted for a period of 4 days. At the last day of the experiment blood samples were collected from a catheter inserted into the EDV and from the femoral vein. The specific activity (SA) in plasma samples was measured to assess if there were any differences between the sampling sites. Total body water and turnover rate were determined according to the dilution rate of tritiated water over time. No statistical differences were shown between the 2 catheters in EDV. However, differences between samples from the femoral vein and EDV occurred in 2 of the seals. Total body water decreased on average ($n = 5$) 1.2 ± 0.1 l during the experimental period. Total rate of efflux was 2035 ± 145 ml \cdot day⁻¹ with a daily net water loss of 297 ± 15 ml. All the seals showed a large extent of mariposia during the 4 days of fasting (1147 ± 153 ml \cdot day⁻¹). It is concluded from this study that sampling site does not affect the SA of ³H₂O in plasma. However, the use of a second catheter is strongly recommended to be sure to avoid any contamination. In addition, it is concluded that sub-adult, fasting hooded seals drink seawater to a significant extent.

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1. Introduction

1.1. Hooded Seal (*Cystophora cristata*)

Hooded seal (*Cystophora cristata*, Erxleben, 1777) is a large phocid that is silver-grey in colour with irregularly distributed black spots covering most of the body and, most often, black head. Males are in average 2.5 meters long and weight around 300 kg while females are significantly smaller measuring in average 2.2 meters and weighting around 200 kg. Adult males have an inflatable nasal sac that fully inflates, covering the front of the face and the top of the head, during the mating season as a specific courtship display (Kovacs, 2008). Blueback is the name given to newborn individuals of this species due to their colour which is kept until the age of 2 years approximately.

Hooded seal is a North Atlantic migratory species with an annual movement cycle associated to drifting pack-ice (Kovacs, 2008). Sergeant (1976) determined the status of the hooded seal in the North Atlantic and described three major sub-populations: one in Newfoundland, the second one in the Davis Strait and the last one around Jan Mayen, Greenland sea (West-Ice) (Figure 1). The name of each population is given by the location of the breeding/whelping patch.



Figure 1. Map showing the distribution of hooded seals (pink-shaded area) (Kovacs, 2008).

Folkow et al. (1996) further described the annual distribution of hooded seals in the Greenland and Norwegian seas. Studies based on satellite-linked transmitters showed that seals perform long and repeated journeys to open waters as far as Faeroe Islands, south Iceland, Bear island and Svalbard. However, in periods other than breeding and moulting, seals spread further out into open sea and for longer periods of time (Folkow et al., 1996; Folkow et al., 2010). Folkow et al. (2010) determined that the overall time spent by a young of the year hooded seal in open ocean amounts to 76 % of its life.

This creates potential challenge for seals regarding to water balance (Folkow & Blix, 1987) and homeostasis since during that 76 % of the time they do not have access to fresh water. In addition, the main food items for hooded seals are fish such as polar cod (*Boreogadus saida*), cod (*Gadus morhua*), herring (*Clupea harengus*), capelin (*Mallotus villosus*), sand eel (*Ammodytes spp.*) or Greenland halibut (*Reinhardtius hippoglossoides*); crustaceans and squid (*Gonatus fabricii*) (Haug et al., 2007). Crustaceans and squid are practically isotonic with seawater (Fetcher, 1939) while fish are hyposmotic ($\sim 400 \text{ mOsm} \cdot \text{l}^{-1}$) related to seawater ($\sim 1000 \text{ mOsm} \cdot \text{l}^{-1}$) (Schmidt-Nielsen, 1979). Therefore, even though all of them provide water, feeding involves a high load of salts for the seals.

1.2. Water balance and homeostasis

Homeostasis is defined as the whole set of auto-regulation systems that leads to the maintenance of the constant composition, properties and normal functioning of an organism (Hill et al., 2012). Regarding water balance, homeostasis means the auto-regulation and proper control of the water pool in an organism. As it is said above (see section 1.1.) this is a challenge for hooded seals when spending several months a year in open seas.

Marine mammals are well adapted to their hyperosmotic environment by possessing physiological mechanisms developed to preserve fresh water and avoid dehydration. Furthermore, some marine mammals, such as hooded seals, have the ability to maintain water balance during extended periods without any access to food or water (fasting periods) which requires even more robust mechanisms to conserve water (Ortiz, 2001).

Hooded seals have five and four pathways to obtain and lose water, respectively (Figure 2).

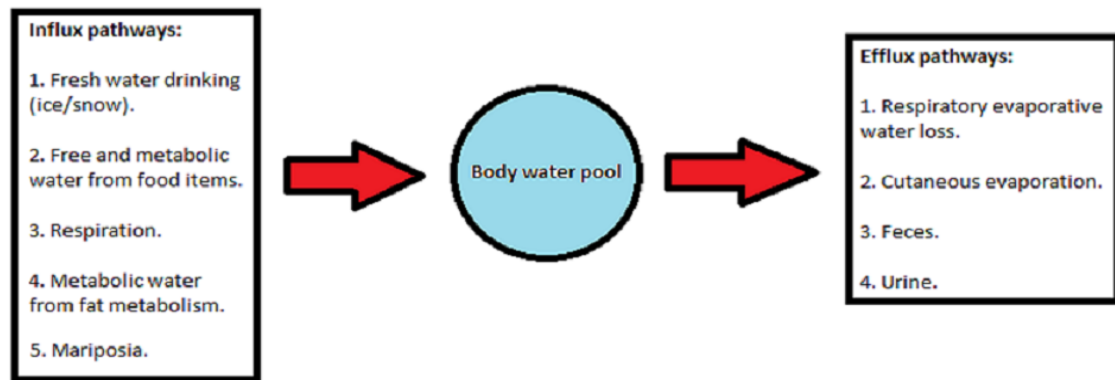


Figure 2. Influx and efflux water pathways of hooded seals.

1.2.1 Fresh water drinking

Fresh water drinking does not appear to be a common practice among phocids (Ortiz, 2001). However, harp seals (*Pagophilus groenlandicus*) have been shown to ingest fresh water daily when given in form of ice cubes in captivity (Renouf et al., 1990) and hooded seals may do so when ice and/or snow are available during breeding and moulting seasons on drifting ice. Anyway, the amount of fresh water consumed by hooded seals has not been measured yet.

1.2.2 Free and metabolic water from food items

Dietary and metabolic water should be sufficient to maintain water balance (Fetcher, 1939; Ortiz, 2001). Depocas (1971), by using tritiated water method, estimated that metabolic water satisfies the needs of full fish-fed seals living in cool environments. According to the data provided in the article, free water in the food counts up to 50 % of the total water flux. Skalstad and Nordøy (2000) estimated that free and metabolic water from the food contributes to an 81 % of the total water turnover in hooded seals (assuming in both cases metabolism to end products). In this case fat metabolism is of great importance since 1.07 grams of water are produced per gram of fat metabolized. However, only 0.40 grams of water are produced per gram of protein (Hill et al., 2012).

1.2.3 Metabolic water from fat reserves

The lipids in seals are primarily stored as subcutaneous fat (blubber) which is one of the main components of a seal. It amounts to 41 % of the body (for the seals in this study) according to the equation $\%TBF = 105.1 - 1.47 \cdot \%TBW$ (Reilly & Fedak, 1990); where TBF is total body fat and TBW is total body water. Blubber functions mainly as a

body stream-liner, insulator, buoyancy adjuster and energy store (Brunborg et al., 2006). As mentioned before, 1.07 grams of water are produced per gram of fat metabolized. This becomes extremely important when there is no access to water or when undergoing fasting periods. Then, metabolic water becomes the only source of water for seals.

1.2.4 Respiratory water influx

Seals are able to exchange water from the air passing through the nasal passage across the nasal mucosa which is a mandatory procedure when nasal heat exchange occurs. Nasal heat exchange mechanism is an efficient water saving mechanism and contributes to water conservation in grey seals (*Halichoerus grypus*) (Folkow & Blix, 1987). Depocas (1971) estimated the water gained by harbor seals (*Phoca vitulina*) to be 93 - 108 ml · day⁻¹. In other studies this value was calculated to be 114 ± 2 ml · day⁻¹ in hooded seals. This amounts to about 5 % of the total water intake (Skalstad & Nordøy, 2000). However, it is been shown that net water flux through respiration is negative, so more water is lost than gained.

1.2.5 Mariposia

Early studies showed that none of the grey seals (*H. grypus*) involved in experiments were observed drinking seawater (Irving et al., 1935). Also Fetcher & Fetcher (1942) determined that marine mammals have not got special adaptations in the intestine so they do not drink seawater. They suggested that water made available in the food is sufficient for excretion, thus, there is no reason to suspect that kidneys of seals are different to those of other mammals. Later studies reinforced this theory suggesting that several observations made on different marine mammals indicate that they do not ordinarily drink seawater and that minimal amounts of seawater are ingested with the food after feces and stomach content analysis. However, they concluded that marine mammals living on invertebrates probably do have a special mechanism to reduce urinary salt concentration (Fetcher, 1939; Depocas, 1971). Also Depocas (1971) claimed that seawater intake is always accidental and related with feeding.

Studies on renal concentrating ability and renal response to a osmotic load have been performed in Baikal seal (*Pusa sibirica*) and ringed seal (*Pusa hispida*). In response to hypertonic salt solution intravenously administered, both urine flow and salt excretion increased markedly. Ringed and baikal seals, thus, are able to eliminate the excess of

salt. Seals are able to concentrate urine up to $2000 \text{ mOsm} \cdot \text{kg}^{-1}$ or above, which serves as a remarkable mechanism for water conservation (Hong et al., 1982).

Tarasoff & Toews (1972) studied the effect of dehydration of harbor seals (*P. vitulina*) and ingestion of distilled water and of seawater. They concluded that mariposia is not an effective mechanism to obtain net gain of water. However, in later studies, harbor seals (*P. vitulina*) have been shown drinking salt water from a pool. When fresh water was offered it was rejected and seals continued to drink salt water. No illness or evidences of vomiting or diarrhoea were observed (Renouf et al., 1990). Skalstad & Nordøy (2000) also demonstrated that harp and hooded seals voluntarily drink seawater with positive effects in water homeostasis. Gentry (1980) showed clear evidences of spontaneous mariposia in a four species of wild pinnipeds through long time-lapse observations.

Recent studies have shown experimental evidences of seawater drinking in hooded and harp seals (*P. groenlandicus*). Taking into account every input and output of water, it was concluded that hooded and harp seals (*P. groenlandicus*) drink $300 \pm 55 \text{ ml} \cdot \text{day}^{-1}$ and $900 \pm 12 \text{ ml} \cdot \text{day}^{-1}$ amounting to 14 % and 27 % of total water turnover, respectively, and that kidneys are capable of excreting the salt load from seawater ingestion (Skalstad & Nordøy, 2000). Further research determined that seawater drinking restores water balance in dehydrated harp seals (*P. groenlandicus*) (How & Nordøy, 2007). Salt water ingestion triggers a highly concentrated urine production ($\sim 2000 \text{ mOsm} \cdot \text{kg}^{-1}$). Seals with no access to seawater experienced an increase in blood osmolality from 331 to 363 $\text{mOsm} \cdot \text{kg}^{-1}$. These values returned to normal when seal were allowed access to seawater. In addition, plasma urea, which is excreted by the use of water, decreased in seals when seawater was available after dehydration from 22 mM to baseline levels (How & Nordøy, 2007; Storeheier & Nordøy, 2001). Mariposia is thus an effective mechanism to gain water.

1.2.6 Respiratory evaporative water loss (REWL)

REWL is dependent on ambient temperature and air humidity. When inhaling cold air it is warmed up and saturated with water on its passage to the lungs. The lungs are at the same temperature as the body core so air rises to $\sim 37.5^\circ\text{C}$ and is 100 % saturated with water vapour. The heat and water needed are transferred from the body, meaning that water and heat would be lost if air is exhaled at those conditions (Hill et al., 2012). Seals have a nasal counter-current heat exchange mechanism that reduces water and heat loss in the exhaled air which has been suggested to be of considerable

importance for thermal and water balance (Folkow & Blix, 1987). Folkow & Blix (1987) determined that exhaled air temperature decreases linearly with decreasing ambient temperature at an ambient temperature below the lower critical temperature. This means that as much as 80 % of the water added to inhaled air can be regained in grey seal (*H. grypus*). In addition, seals can perform apneic breathing in order to reduce REWL. It has been estimated that in northern elephant seals (*Mirounga angustirostris*) REWL is reduced by 41 % when using this mechanism (Lester & Costa, 2006). However, nasal water exchange has not been considered an effector mechanism for water conservation in grey seals (*H. grypus*) since it did not vary at all under conditions where water balance was compromised (Skog & Folkow, 1994). Despite of this, reabsorption of water at the nasal mucosa is an important mechanism to reduce the respiratory water loss.

1.2.7 Cutaneous evaporation

Seals have a very low integumentary permeability to water (Hill et al., 2012). Losses through cutaneous evaporation are generally considered negligible in phocids due to a lack of sweat glands (Lester & Costa, 2006). Since hooded seals live in cold environments they do not need to evaporate water for the regulation of body temperature (Irving et al., 1935). However, even though this issue has been put aside, it must be taken into account. Active sweating on the flippers has been reported in California sea lions (*Zalophus californianus*) amounting to 16 % of heat produced under hot conditions, while the highest evaporative rate was $152 \text{ g} / \text{m}^2 \cdot \text{hour}$ (Matsuura & Whittow, 1973). Ortiz et al. (1978) stated that REWL and cutaneous evaporation must be the predominant ways regarding to water loss in elephant seal pups (*M. angustirostris*). Later studies have shown that cutaneous evaporation may account for up to 34 % of total daily efflux in fasting grey seals pups (*H. grypus*) (Nordøy et al., 1992).

1.2.8 Feces

Water loss by feces production is very limited and negligible, having been measured at $20 \text{ grams} \cdot \text{day}^{-1}$ in fasting northern elephant seal (*M. angustirostris*) pups (Lester & Costa, 2006). In addition, feces during fasting are not or nearly not produced.

1.2.9 Urine

Urine production is the principal water efflux pathway for seals, amounting to 250 ml · day⁻¹ in grey seal pups (*H. grypus*) (Nordøy et al., 1992). When fasting, grey seal pups (*H. grypus*) reduce the urine production to a minimum (around 100 ml · day⁻¹) (Nordøy et al., 1992). In addition, urine osmolality can be increased up to twice as high the seawater concentration in some species, such as harp seal (*P. groenlandicus*) (How & Nordøy, 2007), which makes them capable to keep water balance during fasting or water deprivation.

1.3. Project purpose

The purpose of this MSc project is to study the water balance and methodological aspects of the use of tritiated water of fasting sub-adult hooded seals. The method, which employs water labeled in its hydrogen, is based on the measurement of the apparent turnover rate of the hydrogen of body water over time (Lifson & McClintock, 1966). The method gives, in addition, the possibilities of obtaining values for water input and output, total body water (TBW) and total water turnover rate. Lifson and McClintock (1966) stated six assumptions to be made when using this method that may provide considerable errors:

1. The animal is in a steady state of body composition: total body water remains constant and also weight and composition of body solids.
2. All rates of intake and output remain constant.
3. All the body water is uniformly labeled and there is no incorporation of tritium of body water into other body constituents.
4. Water is the only form in which tritium is lost from the body.
5. The specific activity (SA) of the tritiated water lost from the body is equal to that of the body water. This assumption also implies that the normal abundance of tritium is the same in all substances involved in the material balance.
6. No water, either labeled or non-labeled, enters the body with inspired air or through the skin.

Under almost any condition some error results from the use of the tritiated water method and under certain circumstances the error may be considerably, particularly with regard to water balance values. For instance, mean absolute error in water output were estimated to range between 3 ± 2 to 14 ± 9 % (Lifson & McClintock, 1966).

Laboratory studies performed by Nagy & Costa (1980) indicated that tritiated water measurements are accurate to within -7 to +4 % in marine mammals. However, under field conditions, the errors may be much greater. Errors could exceed ± 50 % in some circumstances or even ± 100 % whether choosing the wrong equation for calculations.

Besides, recent studies suggested that venous sampling site (whether from the extradural intravertebral vein (EDV) or from the flipper vein) may influence the dilution rate of tritiated water in hooded seals (Jakobsen, 2011; Verlo, 2012) and thus the calculations of TBW and total turnover rate.

The main focus of this study is thus to investigate in detail the effect of the sampling site when measuring the dilution rate of tritiated water. The specific activity (SA) of plasma collected will be used to estimate potential differences due to the sampling site. In addition, blood samples will be collected over time to best estimate the equilibration time as well as water balance and rate of seawater intake during 4 days of fasting. Nevertheless, the other potential errors given by the assumptions described above will be taken into account in order to make accurate estimations of TBW and total turnover rate.

2. Material and Methods

2.1. Animals

Five hooded seal pups were caught in March 2012 in the West-Ice between Greenland and Jan Mayen (~72°N, ~16°W) and transported on board RV Helmer Hanssen to the Department of Arctic Biology (AAB) at the Arctic University of Norway. The seals were kept at AAB for 20 months before the experiments in a 42000 litres seawater tank with a continuous water supply of 50 litres · min⁻¹ and exposed to simulated (70°N) seasonal variation in photoperiod. Room and water temperature were ~6.5°C and ~6°C, respectively, during the experimental period, from mid-November to mid-December, 2013.

Seals were fed 1 or 2 times a day with between 2-3 kg · day⁻¹ of recently thawed herring. Herring was stored in 20 kg blocks frozen at -20°C and left overnight to thaw in running water before feeding the animals. Also a vitamin complex (SEA TABS Antioxidant Vitamin Supplement formulated for marine mammals III) (Table 1) was administered with each meal as suggested by Blix et al. (1973).

SEA TABS® MA Antioxidant Vitamin Supplement formulated for MARINE ANIMALS	
Per Tablet	Amount
Vitamin A (Retinol Acetate)	5,000 I.U.
Vitamin D3 (Cholecalciferol)	400 I.U.
Vitamin C (Calcium Ascorbate)	10 mg.
Vitamin E (d-Alpha Tocopheryl Succinate)	50 I.U.
Vitamin B1 (Thiamine Mononitrate)	250 mg.
Vitamin B2 (Riboflavin)	2.0 mg.
Vitamin B3 (Niacinamide)	6.0 mg.
Vitamin B6 (Pyridoxin HCl)	2.0 mg.
Folic Acid	500.0 mcg.
Vitamin B12 (Cyanocobalamin)	10.0 mcg.
Vitamin B5 (D-Calcium Pantothenate)	5.0 mg.
D-Biotin	200.0 mcg.
Kelp Plant Extract (<i>Laminaria digitata</i>)	20.0 mg.
CLA (Conjugated Linoleic Acid)	10.0 mg.
Inositol	5.0 mg.
Lycopene	300 mcg.
Lutein	250 mcg.

Table 1. Formula of the tablets given to the seals within each meal. I.U. is International Units, mg is milligrams and mcg is micrograms.

The weight of the seals at the beginning of the experimental period are present in the following table (Table 2):

Table 2. Weight of the seals used in the experiment at t = 0 (after 24 h fasting).

Animal	Weight (kg)
Seal #1	103.5
Seal #2	87.6
Seal #3	96.4
Seal #4	83.4
Seal #5	87.5

The animals were accustomed to human presence. Seals were fasted during the experimental period of 5 days.

2.2. Experimental protocol

Experiments were carried out in November-December 2013 following the protocol described below (Figure 3).

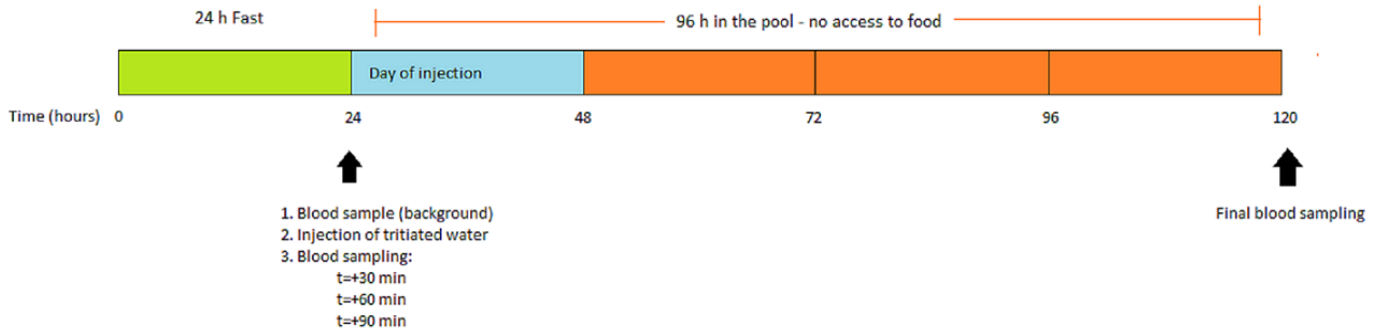


Figure 3. The experimental protocol used.

Seals were fasted 24 hours prior to beginning of the experimental period. Experiments were performed in the following 4 days, still fasting, in a total experimental period of 5 days.

The pool was drained and seals captured and administered a 1.0 ml intramuscular injection of the sedative tiletamine-zolazepam ($50 \text{ mg} \cdot \text{ml}^{-1}$ tiletamine, $50 \text{ mg} \cdot \text{ml}^{-1}$ zolazepam) (Zoletil Forte Vet, Reading, L'Hay-Les-Roses, France). After 10 minutes sedation made effect on the animals. Then, seals were captured using a big specially designed nylon bag, weighted (Dini Argeo MCWNT 1RF-1; 10-150 kg, accuracy $\pm 0.5 \text{ kg}$;

Spezzano di Fiorano, Italy) and restrained on a board. The head was covered with a textile bag to keep the seals calm by preventing vision.

Two catheters (Selacon-T™ 16G/1.70x160 mm, The Hague, The Netherlands) were inserted into the EDV at the level of the lumbar vertebra and sutured when needed. At $t = 0$, blood was collected for measuring background activity of tritium and injection dose (ID) estimation. ID will be estimated experimentally using uncontaminated plasma by adding to it a certain amount of tritiated water (see section 2.4.1). Then, tritiated water (between $6.5 \cdot 10^5$ and $1.3 \cdot 10^6$ CPM \cdot kg⁻¹) was injected through catheter #1 (EDV1, further up in the spine). Tritiated water was prepared as a solution of 30 μ l of Tritium (3.7 GBq \cdot ml⁻¹; Lot. No 2656-135, 1989. Kjeller, Norway) in 50ml saline (B. Braun 9 mg \cdot ml⁻¹ NaCl, Melsungen, Germany).

At intervals +30, +60 and +90 minutes after injection, 30 ml blood was collected from each catheter (EDV1 and EDV2) into vacutainers (BD Vacutainer® LH170 I.U. 10.0 ml, Plymouth, UK) to determine potential differences between catheters and estimate total body water (TBW). Additional 10 ml blood were collected with a vacutainer from the flipper vein at $t = +60$ to determine differences due to sampling site. Tubes were turned upside down few times to avoid coagulation. Catheters were gently flushed with saline (9 mg \cdot ml⁻¹ NaCl, Melsungen, Germany) after sampling to avoid contamination and obstruction by clotting of blood. Then, catheters were withdrawn, injection sites were disinfected with chlorhexidin (5 mg \cdot ml⁻¹) and seals reintroduced into the pool. After 96 hours in the pool, seals were recaptured as described above and 30 and 10 ml blood sampled from the EDV (catheter) and the flipper vein (vacutainer), respectively, to determine water turnover rate.

Blood was centrifuged (Kubota KS-8000, Tokyo, Japan) right after sampling for 10 min at 2000 rpm and plasma transferred to 4.5 ml cryotubes (WWR INTERNATIONAL, Norway) and stored in a -80°C freezer until analysis.

2.3. Plasma analysis

All plasma samples were analyzed for specific activity (SA) in a Liquid Scintillation Counter (LSC) (Packard 1900 TR Beta-Teller Liquid scintillation Analyzer, Oslo, Norway). Uncontaminated plasma, collected at $t = 0$, was used to estimate injected dose as explained in section 2.4.1. Before using uncontaminated plasma to determine injected dose several counts were performed to assure that background level of tritium in

plasma was negligible. Background level was in average $69 \pm 54 \text{ cpm} \cdot \text{ml}^{-1}$, ranging from 22 to 128 $\text{cpm} \cdot \text{ml}^{-1}$.

Plasma samples were left overnight to thaw. They were then transferred to glass tubes and 3000 μl of each sample pipetted to another glass tube. Perchloric acid (HClO_4 : 70 %; Sigma-Aldrich Química S.L., Tres Cantos, Madrid) was added to plasma (200 μl $\text{HClO}_4 \cdot \text{ml}^{-1}$ plasma) in order to deproteinize the plasma so quenching due to protein precipitation (25 %) is avoided (Bray, 1960). The mixture was mixed using a vortex mixer (Fisons Whirlmixer TM, England) and then centrifuged for 15 minutes at 2500 rpm. A volume of 500 μl of supernatant was then transferred to a 20 ml liquid scintillation vial together with 9.5 ml of scintillation fluid Ultima Gold™ (LSC cocktail, Sigma-Aldrich Química S.L., Tres Cantos, Madrid). Three or four replicates were prepared of each sample (depending on the volume of supernatant available). LSC was set to count ^3H samples during 10 minutes per sample and 1 cycle.

2.4. Estimating TBW and water turnover rate

2.4.1 CPM correction

Results from LSC were first corrected according to the total water volume of the final sample. Thus, conversion factors for plasma and HClO_4 water content were calculated.

Plasma water content was estimated by drying 1 ml of plasma in a Petri dish in an oven at 60°C (Temarks TS 4115, Bergen, Norway). Plasma was weighted before and after 24 hours drying and water content calculated by subtracting weight at $t = 1$ to that at $t = 0$. This procedure was repeated 6 times and the average calculated. Plasma water content was estimated to be $92.0 \pm 0.9 \%$.

Perchloric acid was 70 % concentrated so it was assumed that the remaining 30 % was water. Then, conversion factors to be applied are next:

$ \begin{array}{r} 3000 \mu\text{l plasma} > 92\% \text{ H}_2\text{O} = & 2760 \mu\text{l H}_2\text{O} \\ 600 \mu\text{l HClO}_4 > 30\% \text{ H}_2\text{O} = & 180 \mu\text{l H}_2\text{O} \\ \hline & 2940 \mu\text{l H}_2\text{O} \end{array} $	Conversion Factor = $2940/2760 = 1.065$
---	---

In addition, only 500 μl of sample was used when counting so the cpm value had to be adjusted to end up with $\text{cpm} \cdot \text{ml}^{-1}$ by multiplying by 2. Thus, the final cpm value was calculated as follows:

$$\text{cpm value} \times 1.065 \times 2 = \text{cpm} \cdot \text{ml}^{-1} \text{ water}$$

The injected dose (ID) was estimated as follows (20-25 replicates). A dilution series was prepared by adding 1 ml from the stock solution to 9 ml of distilled water. This procedure was repeated to end up with a dilution 1:100. According to previous experiments, 90 $\mu\text{l} \cdot \text{ml}^{-1}$ plasma from the dilution 1:100 was the right amount to obtain values of cpm within the range of maximum efficiency of the LSC.

Thus, 360 μl were added to 4 ml of uncontaminated plasma and 800 μl of perchloric acid (HClO_4 : 70 %) were added to deproteinize the plasma. Five hundred μl of this mixture were counted in the LSC. As many replicates as possible (20-25) were performed to best estimate the ID. The following conversion factor was used:

4000 μl plasma	> 92% H_2O	=	3680 μl H_2O	Conversion Factor = 4280/500 = 8.56
800 μl HClO_4	> 30% H_2O	=	240 μl H_2O	
360 μl stock sol.	> 100% H_2O	=	<u>360 μl H_2O</u>	
			4280 μl H_2O	

The cpm value was adjusted to the 4 ml of plasma used at the beginning:

$$\text{cpm value} \times 8.56 = \text{cpm values in 4 ml of plasma}$$

With this value the SA in the stock solution was calculated by going back through the dilution rates:

$$\text{cpm value} \cdot 4 \text{ ml}^{-1} \text{ plasma} \times (10000/360) \times 10 = \text{cpm} \cdot \text{ml}^{-1} \text{ in stock solution}$$

where, 10000 is the volume in μl of the dilution number 2, 360 is the volume in μl taken from that dilution and 10 is the dilution rate between the first and second step in the dilution series.

2.4.2 TBW estimation

TBW (ml) may be calculated by using the following equation (Nordøy et al., 1992):

$$TBW = i. d. / SA_0$$

where; i.d. is the injected dose (cpm) and SA_0 is the specific activity ($\text{cpm} \cdot \text{ml}^{-1}$) of tritiated water at equilibrium. SA_0 was calculated using both a mean value of SA measured at $t = +60$ and $+90$ minutes and the value given by the intersection with x-axis when samples at these intervals and at $t = \text{END}$ were fitted in a linear regression model. The measure at $t = +30$ minutes was left aside because it was considered to be too close to the injection time that equilibrium could not be reached yet. At this point, before the equilibrated state, SA decreases so rapidly that the error introduced may be very large. Thus, potential errors due to methodology could be assessed. Only samples from EDV 2 were used to this purpose since it was the one surely uncontaminated. Further calculations regarding to turnover rate were performed only with the TBW calculated from the intercept value of the linear regression model. TBW at $t = \text{END}$ was calculated assuming no changes in percentage of body water so water content calculated at $t = 0$ was adjusted to the final body mass.

2.4.3 Water turnover rate

Nagy & Costa (1980) provided different equations in order to calculate total water turnover rate. In this case, body water volume was assumed to change linearly since the short period that seals were fasting do not compromise water balance for these animals. Ortiz et al. (1978) reported elephant seal pups fasting up to 10 weeks maintaining homeostasis and Nordøy et al. (1992) evidenced that grey seal pups endure 52 days of fasting and water deprivation with minimal dehydration. Then, total water turnover rate was calculated using equations number 4 and 6 from Nagy & Costa (1980) described below:

$$\frac{\text{ml H}_2\text{O influx}}{\text{kg day}} = \frac{\text{ml H}_2\text{O efflux}}{\text{kg day}} + \frac{2,000(W_2 - W_1)}{t(M_1 + M_2)} \quad (6)$$

$$\frac{\text{ml H}_2\text{O efflux}}{\text{kg day}} = \frac{2,000(W_2 - W_1)\ln(H_1^* W_1 / H_2^* W_2)}{(M_1 + M_2)\ln(W_2 / W_1)t} \quad (4)$$

where; W is the body water volume in millilitres, M is the body mass in grams, H is the specific activity of tritiated water in $\text{cpm} \cdot \text{ml}^{-1}$, t is the time in days between the start and the end of the experiments and subscripts 1 and 2 represent initial and final values, respectively. From the total turnover rate, water fluxes associated with the different influx/efflux pathways were calculated.

2.4.3.1 Metabolic water

Basal metabolic rate was calculated using Kleiber's equation (1961) $\text{BMR} = 70 \cdot \text{BW}^{0.75}$ where BMR is basal metabolic rate in $\text{Kcal} \cdot \text{day}^{-1}$ and BW is body weight in kg. The value obtained is multiplied by 2 to estimate field metabolic rate (FMR) since animals are actively swimming during the experimental period.

Assuming that all the energy is obtained from metabolizing fat and that the energy content of fat is $9.3 \text{ kcal} \cdot \text{g}^{-1}$ (Hill et al., 2012) the amount of fat (grams) metabolized per day was calculated by dividing FMR by $9.3 \text{ kcal} \cdot \text{g}^{-1}$. According to Hill et al. (2012) 1.07 grams of water are produced per 1 gram of fat metabolized. In order to calculate daily water obtained via metabolism grams of fat metabolized are multiplied by 1.07.

2.4.3.2 Water influx via respiration

Respiratory minute volume (RMV) was calculated to estimate water input via respiration using the following equation from Folkow & Blix (1987):

$$\text{RMV} = 0.042 \cdot \text{FMR} + 0.119 (\text{l} \cdot \text{min}^{-1} \cdot \text{kg}^{-0.75})$$

where; RMV is respiratory minute volume in $\text{l} \cdot \text{min}^{-1} \cdot \text{kg}^{-0.75}$ and FMR is field metabolic rate in Watts. Field metabolic rate was calculated from Kleiber's equation as explained in section 2.4.3.1. This value had to be converted to $\text{Watt} \cdot \text{kg}^{-0.75}$ to fit in Folkow & Blix's equation (1987) as follows:

$$\text{FMR} (\text{kcal} \cdot \text{day}^{-1}) \cdot 0.0484 \cdot \text{BM}^{0.75} = \text{FMR} (\text{Watts} \cdot \text{kg}^{-0.75})$$

where; 0.0484 is the conversion factor to convert $\text{kcal} \cdot \text{day}^{-1}$ to Watts and BM is body mass. RMV was then calculated and converted to $\text{l} \cdot \text{day}^{-1}$.

Assuming a 100 % saturation of water vapour in air (due to low ambient temperature and seals breathing just above pool water) and knowing the ambient temperature the amount of water in air was calculated. Firstly, P_{H_2O} was calculated:

$$P_{H_2O} = n \cdot R \cdot T / V$$

where; P_{H_2O} is partial pressure of water vapour in mm Hg, n is moles of water in saturated air at a given temperature (0.40663 moles), R is the gas constant ($0.0821 \text{ l} \cdot \text{atm} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$), T is the ambient temperature in Kelvin and V is volume (1000 litres). Using the same formula, the volume of 1 mole of water vapour was also calculated at 1 atmosphere (V_{mol}). Then, water influx via respiration was calculated as follows:

$$W_i = \text{RMV} \cdot P_{H_2O} / 760$$

where; W_i is inspired water in $\text{l} \cdot \text{day}^{-1}$, RMV is respiratory minute volume in litres $\cdot \text{day}^{-1}$, P_{H_2O} is partial pressure of water vapour in mm Hg and 760 is the standard pressure in mm Hg. Then,

$$W_{\text{inf}} = W_i / V_{\text{mol}} \cdot 18$$

where; W_{inf} is the amount of water entering the body in $\text{ml} \cdot \text{day}^{-1}$, V_{mol} is the volume of 1 mole of water vapour at a given temperature in $\text{l} \cdot \text{mol}^{-1}$, and 18 is the molar mass of water in $\text{grams} \cdot \text{mol}^{-1}$.

2.4.3.3 Sea water drinking

The extent of mariposia was estimated by subtracting metabolic water influx and respiratory influx from total water input.

2.4.3.4 Respiratory evaporative water loss (REWL)

Respiratory evaporative water loss was calculated following the same procedure as when calculating water influx via respiration (see section 2.4.3.2). Temperature of exhaled air was adjusted according to Folkow & Blix (1987) to be 29°C.

2.4.3.5 Cutaneous evaporation (CEWL)

Cutaneous evaporation was calculated from data available for gray seals (*H. grypus*). According to Nordøy et al. (1992), 34 % of total daily water efflux in a 39.5 kg grey seal is due to cutaneous evaporation amounting to $3 \text{ ml} \cdot \text{day}^{-1} \cdot \text{kg}^{-1}$. This value was used to determine cutaneous evaporation in the hooded seals used to the present study by extrapolating to the weight of the hooded seals.

2.4.3.6 Urine and feces

Water loss in feces was assumed to be negligible in fasting seals (Nordøy et al., 1992; Storeheier & Nordøy, 2001). Urine production was calculated by subtracting respiratory evaporative water loss (REWL) from total water turnover rate (assuming 100 % of urine to be water).

2.4.4 Fractionation

Isotope fractionation occurs since tritiated water (heavier; $^3\text{H}_2\text{O}$) evaporates slower than unlabeled water (lighter) so specific activity in water lost through respiration does not equal that in body water (Nagy & Costa, 1980). Water turnover rate was corrected for fractionation as follows:

$$r_{\text{H}_2\text{O}} = f_i \cdot r_G + r_L$$

where; $r_{\text{H}_2\text{O}}$ is the corrected turnover rate in $\text{ml} \cdot \text{day}^{-1}$, f_i is the fractionation factor, r_G is the gaseous fraction of turnover in $\text{ml} \cdot \text{day}^{-1}$ and r_L is the liquid fraction of turnover in $\text{ml} \cdot \text{day}^{-1}$. The fraction of turnover subjected to fractionation was calculated as the percentage of gaseous fraction of turnover over the total turnover rate. The fractionation factor introduced in the equation was 0.93 (Sepall & Mason, 1960).

2.4.5 Exchange

The hydrogen atoms of water molecules disassociate rapidly and can freely exchange with hydrogen of organic molecules or become associated with non-aqueous compounds. This introduces an error that must be taken into account (Nagy & Costa, 1980). TBW was not corrected for exchange due to the short equilibration time

decided. However, water turnover rate must be corrected as follows because exchange may be considerable after 4 days:

$$\frac{(r_{H_2O})_{\text{calc}}}{r_{H_2O}} = \frac{r_{H_2O}^R + r_{H_2O}^E + jr_{H_2O}^I}{r_{H_2O}^R + r_{H_2O}^E + r_{H_2O}^I}$$

where; $(r_{H_2O})_{\text{calc}}$ is the uncorrected value of turnover in $\text{ml} \cdot \text{day}^{-1}$, r_{H_2O} is the corrected turnover rate in $\text{ml} \cdot \text{day}^{-1}$, $r_{H_2O}^R$ is water lost from all remaining routes in $\text{ml} \cdot \text{day}^{-1}$, $r_{H_2O}^E$ is water lost via expiration in $\text{ml} \cdot \text{day}^{-1}$, $r_{H_2O}^I$ is water entry via inspiration in $\text{ml} \cdot \text{day}^{-1}$ and j is the ratio of the SA of inspired air to that of the body water ($j = 0$ because the SA of the air is negligible under normal conditions).

2.5 Statistics

Specific activity, TBW and turnover rates of the different seals were analyzed by Student's t test for either independent or paired samples as necessary. Normality tests were done in all the cases and Levene's analysis for homoscedasticity performed when Student's t test for independent samples was used. When Levene's test was significant (differences between variances), a Welch's test was done instead. Those sets of samples where normality was rejected were analyzed by a Mann-Whitney's U test. Significant differences were determined when $p < 0.05$; CI:95 %. Average values are presented as $\bar{x} \pm \text{SD}$ (standard deviation).

3. Results

Body mass was recorded at the beginning and at the end of the experiment. Specific activity (SA) of tritiated water was estimated at $t = 0$ and $t = \text{END}$ from different sampling sites and differences among them, TBW and turnover rate estimated afterwards. Equilibrium for tritiated water distribution was estimated in 3 different ways, described below, to elucidate the best option.

3.1 Daily weight loss

Body mass of seals at $t = 0$ was on average 91.7 ± 8.1 kg (range 83.4-103.5 kg). Mean body mass at $t = \text{END}$ was 88.8 ± 8.2 kg (range 80.2-100.6 kg). Average decrease of body mass was 3.1 ± 0.4 % amounting to 2.8 ± 0.2 kg (range 2.6-3.2 kg) or 0.7 kg \cdot day⁻¹. All the seals were shown to lose weight in a similar manner and rate (Figure 4).

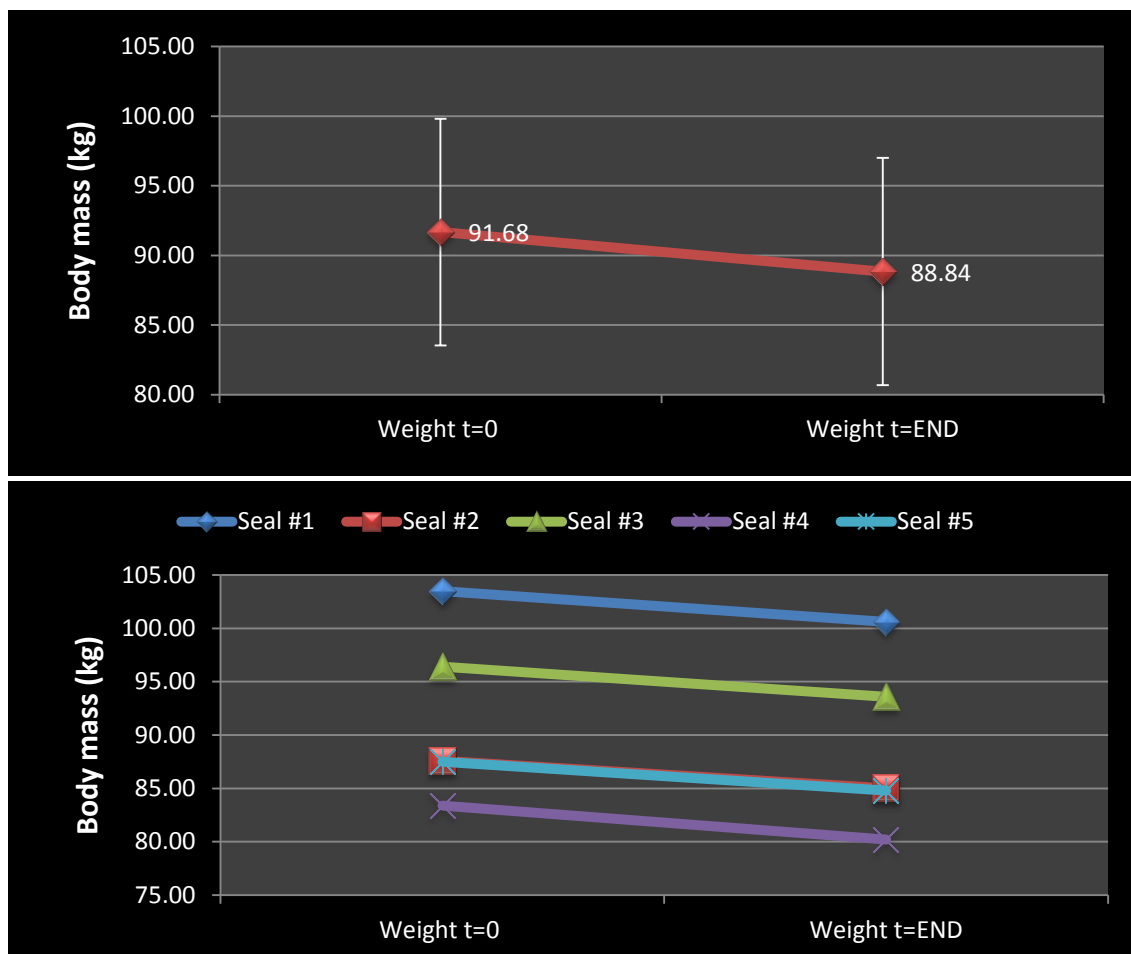


Figure 4. Average weight of seals (n=5) at $t=0$ and $t=\text{END}$ with SD (above) and weight of each seal at $t=0$ and $t=\text{END}$ (below).

3.2 Sampling site variation

Values of SA of $^3\text{H}_2\text{O}$ were used to estimate potential differences due to sampling site. Both variation between the two catheters inserted into the extradural vein (EDV 1 and EDV 2) and between them and samples taken from the flipper vein were analyzed. In both cases, absolute values of cpm, without any correction, were used since, in order to estimate differences, those are not required due to all the samples taken into account in each comparison were subjected to the same treatment.

Seals #1, #2, #3 and #5 were used to estimate potential differences between EDV 1 and EDV 2. Seal #4 was rejected due to the catheter located in the upper part of the spine (EDV 1) had to be taken away because it got blocked.

No statistical differences were shown between catheters EDV 1 and EDV 2 at any time (+30, +60 and +90 min) (Figure 5).

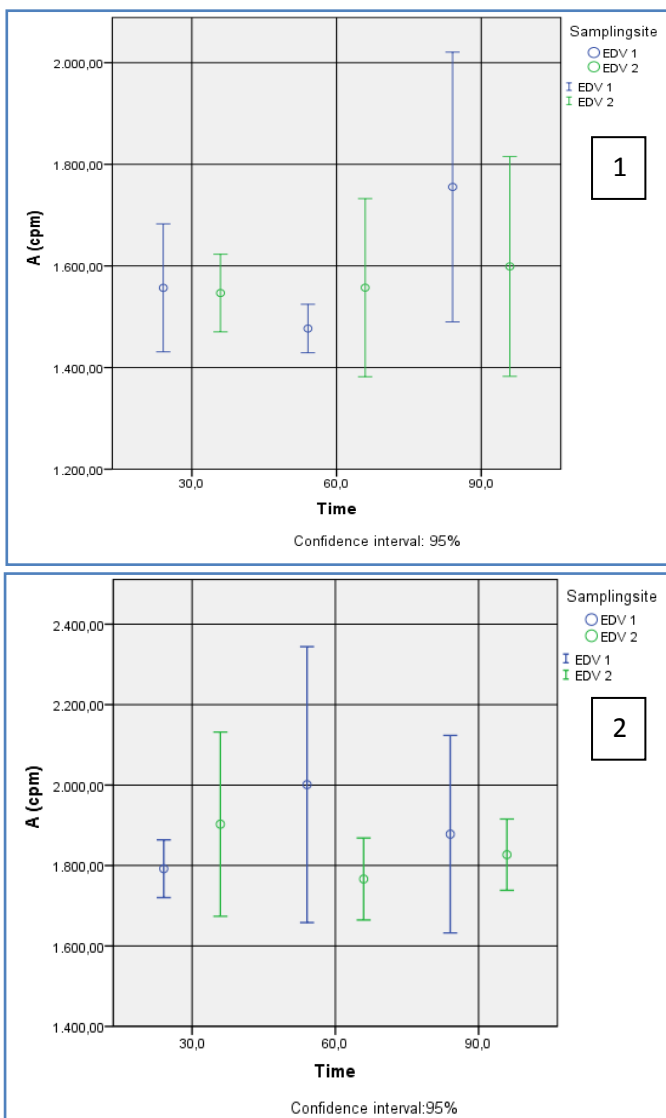
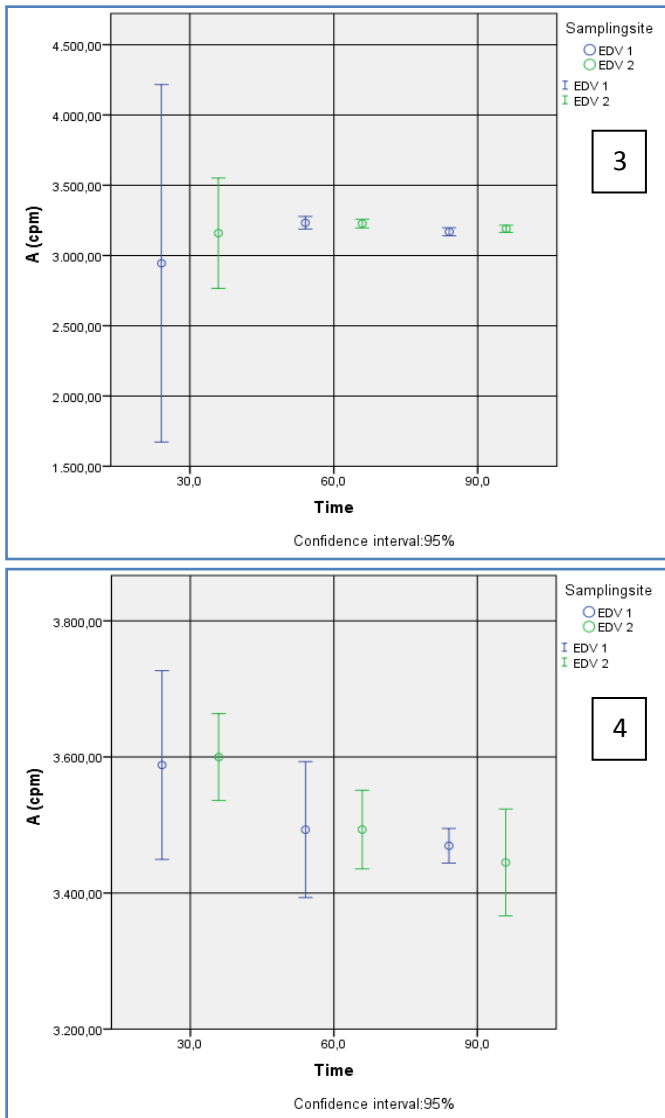


Figure 5. Mean activity values directly from scintillation counting in cpm (A) (circles) and standard deviation (lines) in cpm of the different samples collected from catheters EDV 1 (blue) and EDV 2 (green) plotted as a function of time (minutes), t = +30, +60 and +90 min.

1. Seal #1.
2. Seal #2.
3. Seal #3.
4. Seal #5

Figure 5. Continued.



However, even though not significant, slight variation was shown between samples from EDV 1 and EDV 2 and along the time in seals #1 and #2 (Figure 6).

Samples for these two seals were stored for 48 hours before analysis in a cool room (~2°C) which may introduced an error due to the rapid decay rate of tritiated water. This unexpected variation did not occur in seals #3 and #5 of which samples were analyzed immediately after having been prepared (Figure 7).

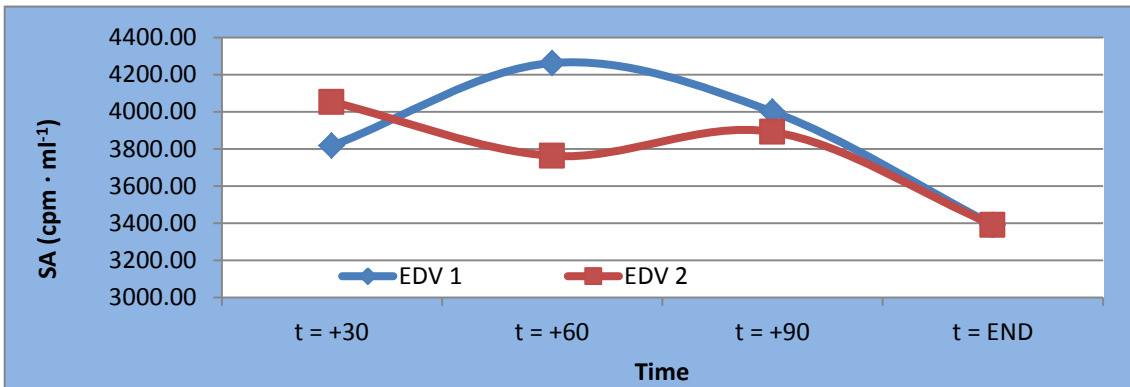
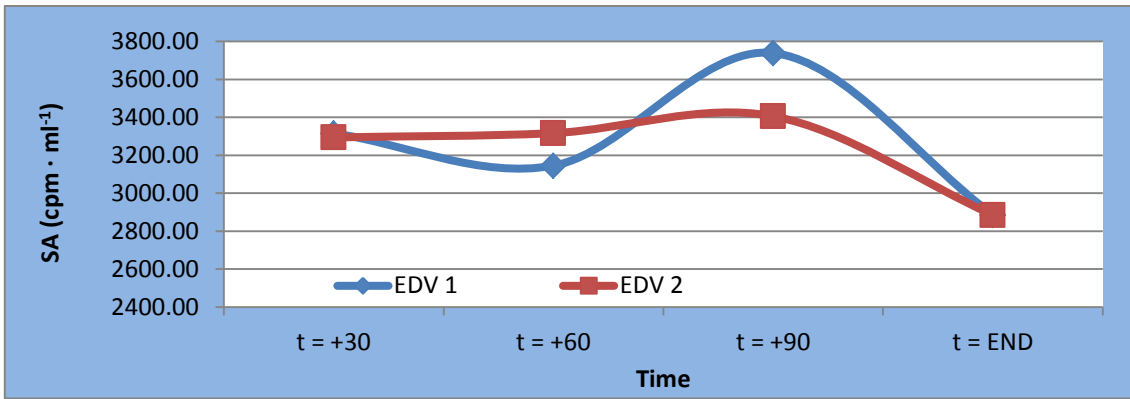


Figure 6. Specific activity (SA) in $\text{cpm} \cdot \text{ml}^{-1}$ of tritiated water plotted as a function of time in seal #1 (above) and #2 (below). $t = +30$ min, $+60$ min and $+90$ min correspond to day 1 of experiment and $t = \text{END}$ to the last sample collected at day 4.

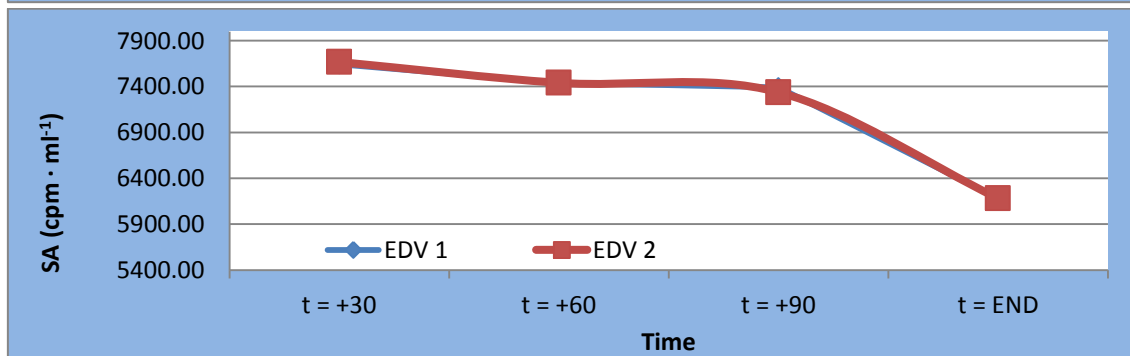
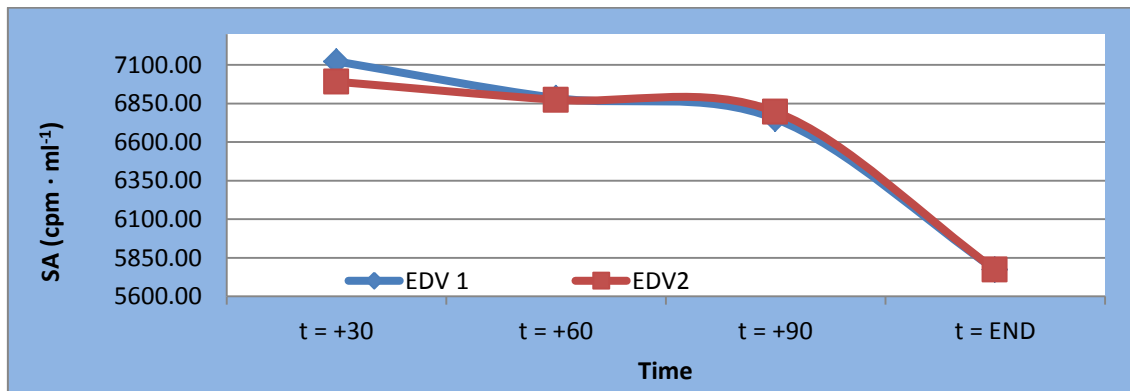


Figure 7. Specific activity (SA) in $\text{cpm} \cdot \text{ml}^{-1}$ of tritiated water plotted as a function of time in seal #3 (above) and #5 (below). $t = +30$ min, $+60$ min and $+90$ min correspond to day 1 of experiment and $t = \text{END}$ to the last sample collected at day 4.

In addition, differences between samples collected from the EDV and the flipper vein (FV) (in the hind flipper) were assessed at t = +60 and t = END. Samples from the EDV 2 were chosen to compare with those from the flipper vein at t = +60. The reason was that this catheter was surely uncontaminated by tritiated water. Comparison was performed 60 minutes after the injection because this time was considered long enough to reach a homogenous distribution of $^3\text{H}_2\text{O}$.

Seals #2 and #3 showed significant variations between samples from the EDV 2 and the FV at t = +60; $p < 0.001$ and $p = 0.008$ (CI:95 %), respectively. Activity measured from the EDV 2 at t = 60 in seal #2 was approximately twice than that measured from the FV. This was most likely an error introduced by adding only half of the volume of plasma from the FV to the counting vial. No other significant variations were shown among the other seals at any time between EDV 2 and FV (Figure 8). Results from seal #4 at t = END are not present because only one sample from FV was available so statistical differences could not be assessed with a sample size of $n = 1$.

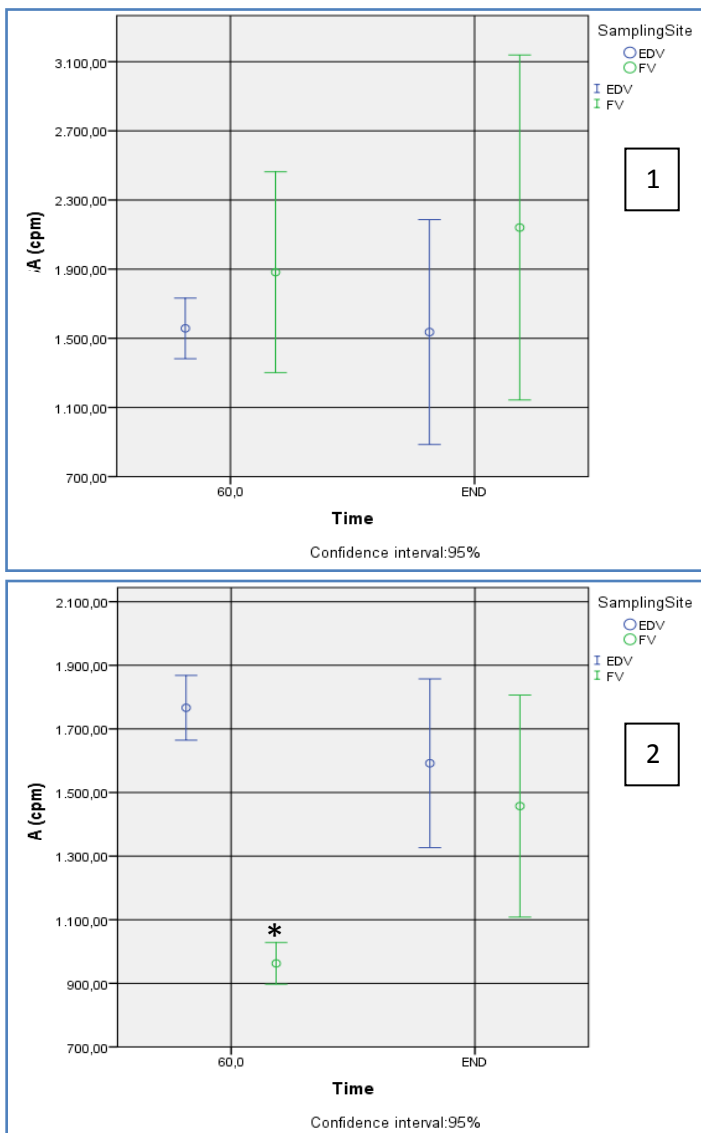
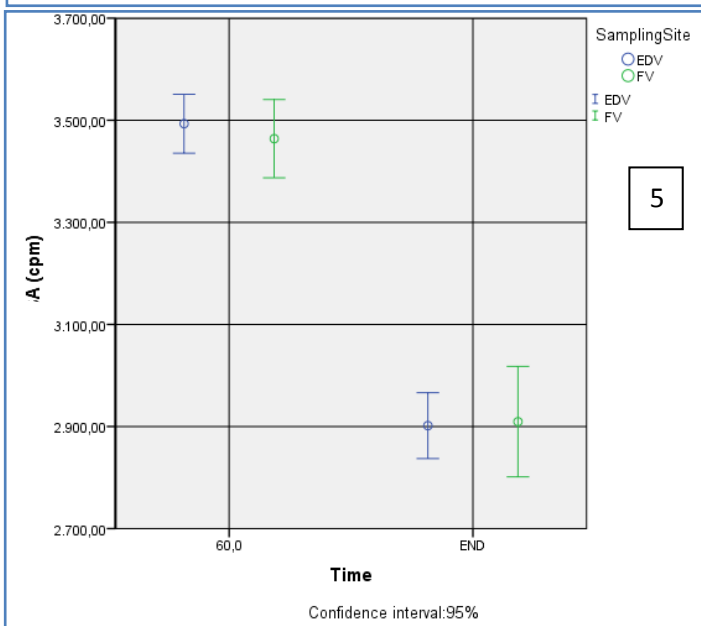
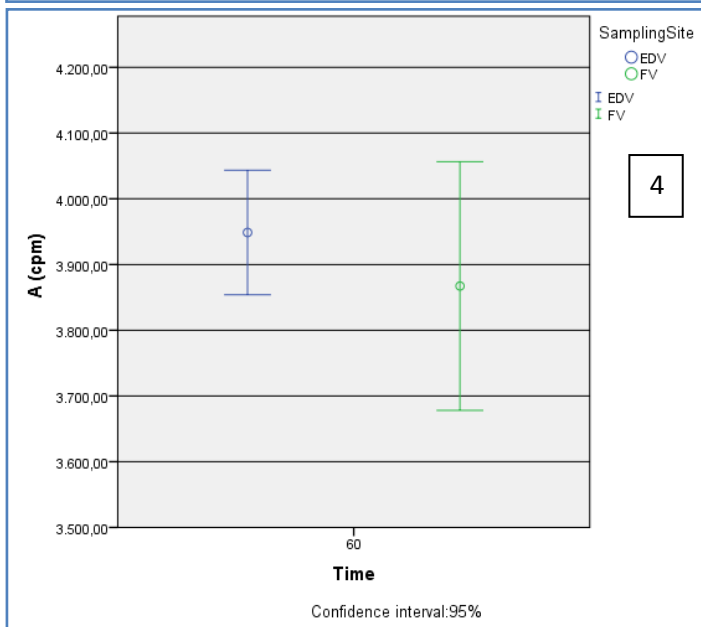
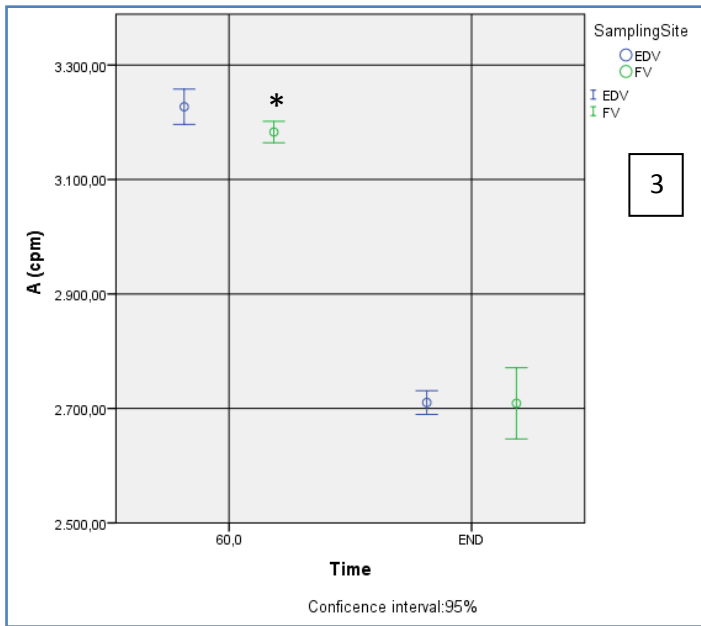


Figure 8. Mean activity values directly from scintillation counting in cpm (A) (circles) and standard deviation (lines) in cpm of the different samples collected from catheter EDV 2 (blue) and FV (green) as a function of time. t = 60 min corresponds to day 1 of experiment and t = END to 4 days after the injection. Asterisks (*) mark those samples where statistically significant differences were shown.

1. Seal #1.
2. Seal #2.
3. Seal #3.
4. Seal #4
5. Seal #5

Figure 8. Continued.



Seal #1 and #2 show an unexpected large variation between samples taken at $t = +60$ and $t = \text{END}$ from FV. Values at the beginning of the experiment are actually higher than those at the end which is, in principle, impossible. The rest of the animals (#3, #4 and #5) show little variation at different sampling sites and an expected variation in SA. Again, this may be related with the storing of samples from seals #1 and #2 which were kept within a plastic box in a room at 2°C during the 48 hours prior to analysis.

3.3 Total Body Water

Data based on isotope dilution rate was used to estimate TBW. Only seals #3, #4 and #5 were utilized to estimate TBW. As mentioned before in section 3.2, samples from seals #1 and #2 were stored 48h before analysis. This might be the reason why SA is very low and the SD very large, compared to these in seals #3, #4 and #5 (Table 3). It was decided to discard those samples when estimating TBW and turnover rate to avoid the possibility of erroneous calculations.

3.3.1 Plasma water content

Table 2 shows the results of the experimental estimation of plasma water content. Average value, 92.1 %, was used then to adjust cpm values to the right water volume present in the samples as described in section 2.4.1.

Table 1. Weights before and after 24 hours drying of 6 plasma samples and their water content as the percentage of the wet weight. Average water content of all 6 samples is presented in the last row.

No. Sample	Net Wet Weight (g) t=0h	Net Dry Weight (g) t=24h	Water Content (%)
1	1.0233	0.0757	92.6024
2	1.0429	0.0994	90.4689
3	1.0553	0.0826	92.1728
4	1.0512	0.0747	92.8938
5	1.0444	0.0759	92.7327
6	1.0670	0.0900	91.5651
Average Water Content \pm SD in Plasma	92.1 \pm 0.9		

3.3.2 Corrected cpm values

Table 3 shows the corrected values of specific activity ($\text{cpm} \cdot \text{ml}^{-1}$) of the whole sample set including those used in the calculations of both TBW and water turnover rate. They were corrected as described in section 2.4.1. Original data set can be found in Appendix 1.

Table 2. Mean values (corrected values) and standard deviations (SD) in $\text{cpm} \cdot \text{ml}^{-1}$ of the SA of $^3\text{H}_2\text{O}$ in plasma samples for each seal at every sampling time and site. Samples taken at $t = +30, +60$ and $+90$ correspond to day 1 of experiment and those taken at $t = \text{END}$ to day 4 of experiment. Asterisks (*) mark those seals that were not taken into account when estimating TBW and water turnover rate.

	Seal #1*		Seal #2*		Seal #3		Seal #4		Seal #5	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
BLANK			21.47	8.65	23.94	3.94	60.30	38.97	104.46	70.95
EDV 1 +30	3316.10	168.42	3817.27	96.16	7122.71	74.15	9230.70	179.56	7642.79	185.77
EDV 2 +30	3294.36	102.21	4052.96	306.62	6990.73	43.24	8628.59	136.25	7667.61	85.45
EDV 1 +60	3145.61	40.80	4262.42	459.10	6885.37	60.22			7440.29	133.74
EDV 2 +60	3317.02	234.58	3762.62	136.39	6873.73	41.34	8410.79	81.19	7440.69	77.29
FV +60	2717.43	1813.22	2050.30	87.44	6779.58	25.15	8237.26	253.28	7378.14	102.74
EDV 1 +90	3739.03	355.31	4000.03	328.75	6750.17	38.30			7389.94	34.00
EDV 2 +90	3406.05	289.47	3891.37	118.76	6795.34	34.10	7965.31	47.12	7337.66	105.06
EDV END	2885.48	492.43	3390.92	355.15	5772.93	27.78	6489.83	53.56	6180.61	86.42
FV END	4527.55	701.46	2328.23	1571.28	5770.12	83.41	6406.02		6197.19	144.82

3.3.3 TBW estimate: linear regression or mean value.

When estimating TBW, SA at equilibrium was assumed to be 1) the mean value of SA measured at $t = +60$ and $+90$ and 2) the value given by the intersection with x-axis when cpm at these intervals and at $t = \text{END}$ were fitted in a linear regression model.

There were no significant differences when calculating TBW using 1) the mean value or 2) linear regression value ($p = 0.842$; $\text{CI}:95\%$; $n = 3$). Difference in TBW regarding to the usage of 1) or 2) was 0.1 l (from 36.8 l to 36.7 l) (Table 4) amounting to 0.15% of TBW. The possible consequence is the overestimate of the efflux and influx rates. However, no significant differences were found in this case either ($p = 0.700$ and 0.700 , respectively). Efflux and influx rates were 0.5% and 0.6% higher, respectively, when using value 2). This amounted to $10 \text{ ml} \cdot \text{day}^{-1}$ in both cases.

Total body water decreased on average $1.2 \pm 0.1 \text{ l}$ ($p = 0.01$) along the experiment, from $36.7 \pm 3.6 \text{ l}$ to $35.5 \pm 3.7 \text{ l}$; $297.6 \pm 15.3 \text{ ml} \cdot \text{day}^{-1}$. TBW at $t = \text{END}$ was calculated

from TBW^{LN} (Table 4). TBW^{LN} was considered the most precise value because it extrapolates the SA to time zero.

Table 3. Total body water in litres at t = 0 and END for seals #3, #4 and #5. Superscripts M and LR mean that the calculations have been done with the mean value and with the linear regression value, respectively. Cumulative water loss is shown in the last column (ΔTBW).

	$TBW^M t = 0$	$TBW^{LR} t = 0$	$TBW^{LR} t = \text{END}$	ΔTBW
Seal #3	40.12	40.07	38.90	1.2
Seal #4	32.92	32.86	31.60	1.3
Seal #5	37.24	37.19	36.04	1.2
Average	36.8 ± 3.6	36.7 ± 3.6	35.5 ± 3.7	1.2 ± 0.1

The linear regression model was also performed using the values at t = +30, +60 and +90 minutes, in order to detect the effect of using the 30 min value. The consequence of this is an underestimation of TBW by about $4.8 \pm 2.3 \%$ (from 39.5 ± 4.1 l to 37.5 ± 4.9 l; n = 3) ($p = 0.628$; CI:95 %) and the overestimation of the efflux and influx rates by $12.3 \pm 2.5 \%$ and $14.5 \pm 2.9 \%$, amounting to $331.3 \pm 93.0 \text{ ml} \cdot \text{day}^{-1}$ ($p = 0.158$; CI:95 %) and $347.2 \pm 102.0 \text{ ml} \cdot \text{day}^{-1}$ ($p = 0.160$), respectively. The differences were still not significant (CI:95%). As mentioned in section 2.4.2 the use of a specific activity too close to the injection time (in this case 30 min) introduces an error.

3.4 Water turnover rate

Total water turnover rate (r_{H_2O}) was estimated to be on average (n = 3) $2036 \pm 145 \text{ ml} \cdot \text{day}^{-1}$, expressed as total daily water efflux. The average total daily water influx was 1738 ± 130 (Figure 9). Efflux was always higher than influx so seals showed a net loss of water of $298 \pm 15 \text{ ml} \cdot \text{day}^{-1}$ which results in a net loss of $1203 \pm 62 \text{ ml}$ during the entire experiment of 4 days (Table 4).

Metabolic water, respiratory water influx and mariposia amounted to 467 (27 %), 123 (7 %) and 1148 (66 %) $\text{ml} \cdot \text{day}^{-1}$ of total daily influx, respectively. On the other hand, cutaneous evaporation, REWL and urine produced amounted to 278 (14 %), 123 (6 %) and 1635 (80 %) $\text{ml} \cdot \text{day}^{-1}$ of total daily efflux (Figure 9 and Table 5).

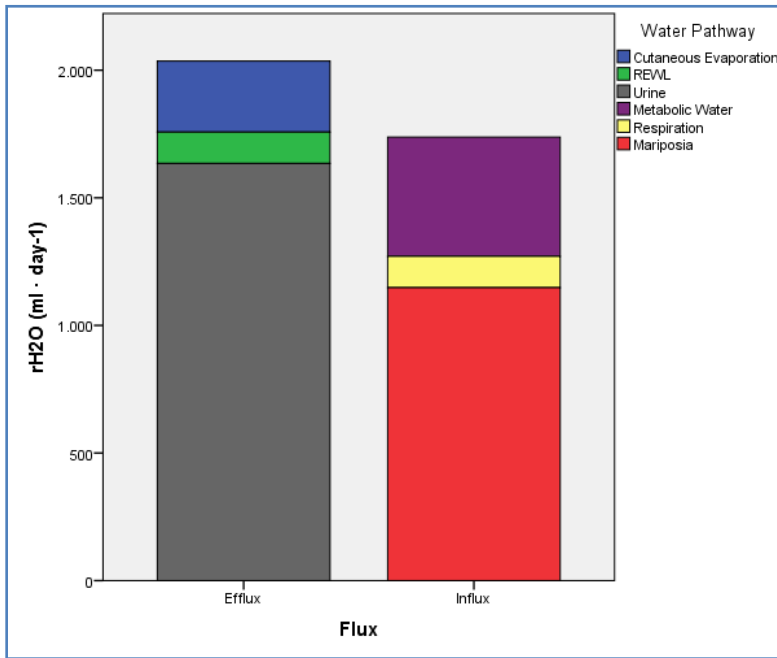


Figure 9. Total water turnover rate (r_{H_2O}) expressed as efflux and influx rates. Besides, cutaneous evaporation, REWL, urine production, metabolic water, water volume lost via respiration and extent of mariposia, of the 3 sub-adult hooded seals, grouped together depending on whether they are influx or efflux pathways. Graph above shows absolute values of turnover in $ml \cdot day^{-1}$ while the one below shows those values as percentage of r_{H_2O} .

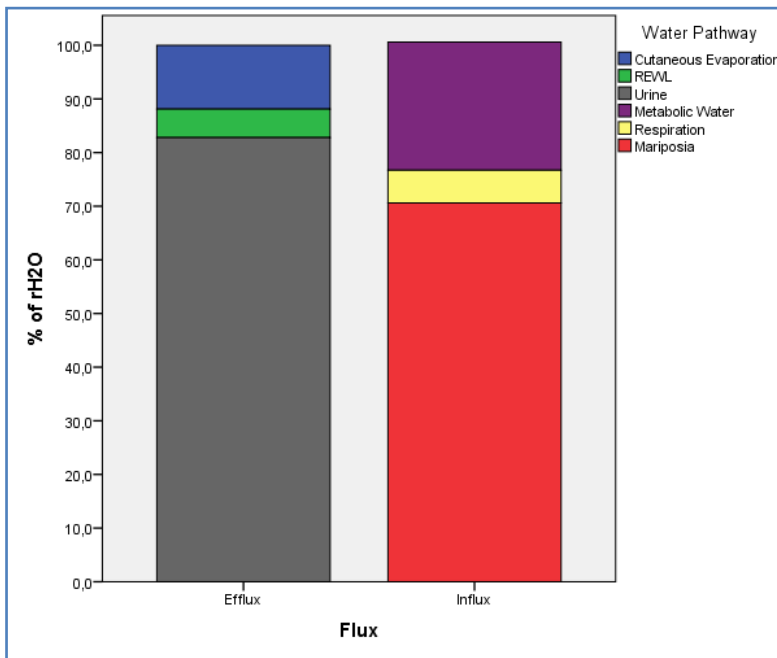


Table 5. Volume in $\text{ml} \cdot \text{day}^{-1}$ of water input and output by the different water pathways, total influx and efflux volume and differences between total influx and total efflux. Average values \pm SD ($n = 3$) are also shown.

	Water pathway	Seal #3	Seal #4	Seal #5	Average ($n = 3$)	SD (\pm)
Influx	Metabolic Water ($\text{ml} \cdot \text{day}^{-1}$)	496	445	461	467	26
	Respiration ($\text{ml} \cdot \text{day}^{-1}$)	131	117	122	123	7
	Mariposia ($\text{ml} \cdot \text{day}^{-1}$)	1052	1325	1065	1148	154
	Total ($\text{ml} \cdot \text{day}^{-1}$)	1679	1887	1648	1738	130
Efflux	REWL ($\text{ml} \cdot \text{day}^{-1}$)	131	117	122	123	7
	Urine ($\text{ml} \cdot \text{day}^{-1}$)	1539	1825	1540	1635	165
	Feces	Negligible	Negligible	Negligible	-----	-----
	Cutaneous evaporation	300	260	273	278	21
	Total ($\text{ml} \cdot \text{day}^{-1}$)	1970	2202	1935	2036	145
Difference influx : efflux ($\text{ml} \cdot \text{day}^{-1}$)		-291	-315	-287	-298	15

Water turnover rate was corrected for fractionation and exchange. The fraction of turnover subjected to fractionation was calculated to be 18 %. So, applying the fractionation factor of 0.93 described in section 2.4.4, fractionation of tritiated water was calculated to underestimate $r_{\text{H}_2\text{O}}$ by 1.3 %. Exchange, otherwise, was calculated to overestimate $r_{\text{H}_2\text{O}}$ by 5 %. Combining fractionation and exchange gives overall overestimation by 3.7 % amounting to $86 \text{ ml} \cdot \text{day}^{-1}$.

4. Discussion

4.1 Daily weight loss

Body mass decreased $3.1 \pm 0.4 \%$ amounting to 2.8 ± 0.2 kg in 4 days of fasting or $700 \text{ g} \cdot \text{day}^{-1}$. Weight loss was partially due to the reduction in TBW (1.2 ± 0.1 l) during the experimental period (1.2 ± 0.1 l). In addition, it has been suggested by an increase in plasma urea in adult harp seals (*P. groenlandicus*) (Storeheier & Nordøy, 2001) that a certain amount of muscle tissue is metabolized at the beginning of a fasting period. This has been seen to be very low, but still important, in grey seal pups (*H. grypus*) before entering into phase III of fasting (Nordøy et al., 1992). Nordøy et al. (1990) calculated the amount of protein catabolized by grey seal pups (*H. grypus*) at the start of the fasting period to be $0.5 \text{ g} \cdot \text{day}^{-1} \cdot \text{kg}^{-1}$ ($20 \text{ g} \cdot \text{day}^{-1}$) covering about 6% of daily metabolic rate. Nordøy et al. (1993) calculated this value to be $0.9 \text{ g} \cdot \text{day}^{-1} \cdot \text{kg}^{-1}$ ($30 \text{ g} \cdot \text{day}^{-1}$) in early phase of fasting in harp seal pups (*P. groenlandicus*). Applying the later value to the current study indicates that about 80 g of weight loss per day can be attributed to protein catabolism. The remaining weight loss was in the current study attributed to fat tissue catabolism.

Dehydration was not likely for this short experimental period (Nordøy et al., 1992; Ortiz et al., 1978) even with a significant decrease in body weight ($p = 0.01$). Nordøy et al. (1992) calculated the daily weight loss in fasting grey seals to be $10.6 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ after 52 days fasting and only minor dehydration was shown. Hooded seals on this study lost on average $7.7 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$.

4.2 Sampling site

Despite no significant differences between EDV 1 and EDV 2 at any time, some slight variation was shown in seals #1 and #2. This is most likely due to the storage of the samples at 2°C for 48 h prior to analysis. When plasma samples containing tritiated water are mixed with the scintillation solvent the isotope starts decaying rapidly (beta pulse decay time = 2-10 nanoseconds) (L'Annunziata, 2003) so they must be analyzed as soon as they are ready. The second plausible hypothesis is that a small fraction of the injected dose remains in the catheter, when this is not properly flushed with saline, contaminating thus subsequent blood samples. This is supported by the lower SD showed in most of the samples collected at the EDV 2 compared to those from the EDV 1 (used for administration of tritiated water).

The procedure was carefully followed and catheters gently flushed with saline after $^3\text{H}_2\text{O}$ dose was administered or blood samples collected (Nordøy et al., 1992; How & Nordøy, 2007). Extreme care with regard to reuse of syringes and keeping the opening of the catheter perfectly cleaned after usage is of high importance in order to avoid potential errors in later estimations.

According with this line of argument the use of one catheter to administer the tritiated water and a second catheter, EDV 2 in this case, for collecting blood samples is strongly recommended to be sure to avoid any contamination. According to Gröning & Rozanski (2003) one Tritium Unit (TU) (1 atom of ^3H per 10^{18} atoms of hydrogen) equals to $0.119 \text{ Bq} \cdot \text{l}^{-1}$. When injecting few mega-Becquerel (MBq) the number of atoms left in the catheter may give huge errors during analysis in a LSC unless they are cleaned properly.

Seals #2 and #3 showed significant differences between samples collected at EDV2 and FV at $t = +60$. However, no other differences were found at any other time or seal. In principle, blood samples from the EDV and FV should be identical, nevertheless, the ability of seals to shut down vascularisation under some circumstances is well known. For instance, when a seal performs a dive it carries out a selective peripheral vasoconstriction. Most of the blood is driven to the brain while the rest of the body must rely on local oxygen stores or anaerobic metabolism (Blix et al., 2010; Blix, 2005). This method is also used to avoid heat loss. In this case, seals reduce peripheral circulation to avoid warm blood going to the flippers, where insulation is least and heat loss largest (Blix, 2005). A reduced blood flow to the flippers may thus prevent a rapid equilibration of tritiated water with total body water as the more central vein, EDV.

Stress is defined as the body's reaction to a change that requires a physical, mental or emotional adjustment or response. It can come from any situation that makes the animal feel frustrated, angry, nervous, or anxious. This behaviour was shown in all the seals at some point during the experiment, mostly when the effect of the sedative decreased with time. Recent studies have shown that levels of cortisol significantly increased when Weddell seals (*Leptonychotes weddellii*) were handled by researchers (Harcourt et al., 2010). Cortisol elevation is a common indicator of stress responses in mammals (Harcourt et al., 2010). Cortisol, among other effects, inhibits vasodilatation (Mangos et al., 2000) which may affect tritiated water distribution through the hind flippers. Administering a light dose of diazepam would ameliorate the cortisol response to handled animals (Harcourt et al., 2010).

In some of the seals used in the experiments a very low surface temperature of the hind flippers was noticed, although not recorded. This indicates low blood flow due to a stressful situation, since room temperature (~6.5°C) would never compromise heat balance in a hooded seal. Folkow & Blix (1987) concluded that the lower critical temperature in grey seals (*H. grypus*) is set at about -11°C. In fact, the experience during the experiment was that blood sampling from the hind flipper was more difficult in those seals with lower flipper surface temperature.

Vasoconstriction due to stress may affect normal distribution of tritiated water. Since the seal is restrained along the entire procedure, from $^3\text{H}_2\text{O}$ dose administration to the last blood sampling (around 2.5 h), during the day one of experiment, this is a very plausible reason why those variations were seen between EDV 2 and FV.

This does not only give an error in later analysis but also makes the sampling much more difficult. Collecting blood samples from the femoral vein when vasculature is nearly non-existing is very difficult and the volume of blood collected often very small and limited. Firstly, a low sampled volume of plasma results in a reduced number of replicates affecting the statistical analysis. Secondly, mistakes or unexpected problems during plasma analysis may end up in gaps in the sample set which makes it impossible to compare some parameters.

In conclusion, the use of a second catheter for collecting the samples seems to be the best option regarding the tritiated water methodology. The only risk of this method is the increased possibilities of hemolysis compared with evacuated tubes used in the flipper vein, probably due to syringe suction during sampling which creates more pressure than a vacutainer (Kennedy et al., 1996). Kennedy et al. (1996) recommend the use of vacutainer adapters to catheters to reduce pressure during the suction of the blood. Frothing of the blood as it passes through the catheter also may provoke hemolysis as well as the pressure applied by pulling and pushing down the plunger of the syringe (Arzoumanian, 2003). However, if a gentle suction is performed, plasma samples obtained from a catheter should be of best quality.

4.3 Total body water

In order to make accurate estimations of total body water (TBW), values of SA measured at $t = +60, +90$ minutes and END were fitted in a linear regression model in order to extrapolate the SA to time zero. During the equilibration time, before any

sample has been collected, some tritium leaves the body since the seal starts eliminating tritiated water via respiration as soon as it enters the body. Therefore, dilution rate is biased. The linear regression model was also generated with the values of SA measured at $t = +30, +60$ and $+90$ minutes. In this case, the calculated SA at $t = 0$ was so large that TBW was calculated to be underestimated $4.8 \pm 2.3 \%$ and water efflux and influx overestimated $12.3 \pm 2.5 \%$ and $14.5 \pm 2.9 \%$, respectively. This suggests that $t = +30$ minutes is too close to the injection time so the value of SA was slightly higher than that at equilibrium. Therefore, it was concluded that equilibrium has not been reached yet at this time.

The last calculation done was with the mean value of SA at $t = +60$ and $+90$. The result varied so little from the value obtained in the linear regression involving $t = +60, +90$ minutes and END. This strongly suggests a low linear fractional turnover rate.

According to the results obtained from the different methods it was concluded that the usage of a linear regression model including values at $t = +60, +90$ minutes and END is the best option to best estimate TBW. The use of a linear regression model to extrapolate a data series is suggested and supported by Chow & Lin (1971). Thus, TBW decreased during the experimental period 1.2 ± 0.1 l, from 36.7 ± 3.6 l to 35.5 ± 3.7 l amounting to $297.6 \pm 15.3 \text{ ml} \cdot \text{day}^{-1}$.

4.4 Water turnover rate

An animal which water balance is in a steady state must match daily water influx and efflux (Depocas et al., 1971). Nagy and Costa (1980) provided equations to be used whether the body water pool remains constant, changes linearly or experiences exponential variation. In addition, potential errors are provided for those cases where the equation is wrongly elected, which could be as high as $\pm 100 \%$.

In this case, the equation for linear changes in TBW was chosen. As mentioned above, TBW of the seals decreased in average 1.2 ± 0.1 l during 4 days of fasting ($297.6 \pm 15.3 \text{ ml} \cdot \text{day}^{-1}$). It is well known that such a short period is unlikely to compromise water balance in seals. This is supported by Nordøy et al. (1992) where grey seals were fasted for 52 days with a daily water efflux of $362 \pm 17 \text{ ml} \cdot \text{day}^{-1}$ and only showed minor dehydration, so the equation for exponential changes was rejected in this study.

Equation for steady state was also rejected because TBW decreased during fasting as a function of time as mentioned before. The low fractional turnover rate mentioned in section 4.3 suggests that the equation for linear changes is the most correct in this case.

According to this line of argument the quantity of water influx and efflux were calculated to be 1737.8 ± 129.9 and 2035.5 ± 145.2 ml · day⁻¹. Metabolic water, respiration and mariposia amounted to 466.97 (26.9 %), 123.20 (7.1 %) and 1147.63 (66.0 %) ml · day⁻¹ of total daily influx, respectively. On the other hand, cutaneous evaporation, REWL and urine produced amounted to 277.63 (13.6 %), 123.22 (6.1 %) and 1634.61 (80.3 %) ml · day⁻¹ of total daily efflux, respectively.

Metabolic water and water influx via respiration compare well to those suggested by Skalstad & Nordøy (2000) and Depocas et al. (1971), respectively. Besides, both values were calculated from well established methods. Metabolic water was calculated based on the Kleiber's equation and respiratory influx from equations provided by Folkow & Blix (1987).

However, the extent of mariposia in hooded seals was estimated to be slightly higher than this in previous publications (Skalstad & Nordøy, 2000). The hypothesis suggested based on this study is that hooded seals increase the extent of mariposia as soon as other sources of water, food in the case of fasting seals, are deprived. This means that, under normal conditions, mariposia is kept below the maximum capacity, which increases when the deprivation of a source of water occurs. This is supported by How & Nordøy (2007) where fasting and dehydrated harp seals were shown to partly restore water balance after 1 l seawater administration which it is a very similar value to that obtained in this study. Skalstad & Nordøy (2000) showed values of mariposia in hooded seal of 10 ml · day⁻¹ · kg⁻¹ (300 ml · day⁻¹) while seals were fed on fresh herring. In that study, free and metabolic water on ingested food and respiratory influx were calculated to be 1750 ml · day⁻¹ and 114 ml · day⁻¹, respectively. It seems that deprivation of food is partly compensated by an increase in metabolic water production from fat reserves (466 ml · day⁻¹) and a small increase in seawater drinking 13 ml · day⁻¹ · kg⁻¹ (1147 ml · day⁻¹) since respiratory water influx (123 ml · day⁻¹) is comparable to that of Skalstad & Nordøy (2000).

The reason may be that producing water from fat reserves involves a certain loss of blubber content with the corresponding loss of energy store and insulating layer which

is of extreme importance for hooded seals living in a cold environment. However, seawater drinking appears not to provide negative physiological effects in hooded (Skalstad & Nordøy, 2000) and harp seals (Skalstad & Nordøy, 2000; Storeheier & Nordøy, 2001) where values of urine and plasma osmolality went back to baseline levels after seawater exposure. This hypothesis reinforces the function of mariposia suggested in early studies, such as providing urinary osmotic space for urea (Wolf et al., 1959). How & Nordøy (2007) showed a clear decrease in plasma urea after seawater administration. In hydrated fasting seals, providing urinary space for urea seems to be the main goal of mariposia. This is the most likely purpose of the mariposia seen in the current study. However, in a state of strong dehydration, the extent of mariposia may be expected to increase considerably to maintain water balance and reduce fat metabolism.

The urine production is also higher than that estimated in other publications (Storeheier & Nordøy, 2001; Skalstad & Nordøy, 2000). This results from the elevated mariposia. The same increase was shown by How & Nordøy (2007) and Storeheier & Nordøy (2001) after seawater administration, since urine is the unique mechanism to excrete the excess of salts in fasting hooded seals.

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Appendix 1

	EDV 1 + 30	EDV 2 + 30	EDV 1 + 60	EDV 2 + 60	FV + 60	EDV 1 + 90	EDV 2 + 90	EDV END	FV END
Seal #1	1615.46	1571.54	1497.40	1458.70	1661.12	1593.60	1602.86	1248.92	2557.54
	1555.20	1558.62	1473.52	1589.10	1694.10	1830.32	1518.14	1195.30	2108.34
	1611.14	1580.50	1459.52	1482.44	1747.94	1644.10	1486.24	1619.84	1756.22
	1445.62	1475.94		1698.90	2427.22	1953.64	1789.10	2080.34	
Seal #2	1822.32	1729.08	2058.58	1730.24	954.14	1964.32	1806.26	1498.54	1619.54
	1817.14	2027.62	1812.84	1811.82	910.02	2025.12	1875.64	1832.74	1382.5
	1803.66	2014.46	2281.10	1695.28	1006.52	1848.00	1757.54	1572.42	1370.22
	1725.46	1840.04	1852.02	1828.62	979.64	1674.36	1868.30	1464.22	
Seal #3	3376.14	3305.38	3199.72	3241.30	3196.80	3178.74	3202.00	2715.62	2748.72
	3348.82	3268.52	3253.60	3219.74	3186.14	3189.24	3178.70	2726.02	2703.12
	1746.12	3272.20	3258.56	3244.32	3180.14	3157.90	3206.02	2697.20	2657.30
	3307.02	2789.92	3218.40	3203.06	3168.52	3150.50	3174.48	2702.34	2726.76
Seal #4	4326.92	4099.14		6309.72	4037.56		3765.16	3040.22	3007.52
	4217.86	4075.40		3977.36	3798.84		3736.52	3061.14	
	4381.56	3978.40		3963.36	3774.22		3744.92	3071.52	
	4408.30	5702.90		3905.46	3858.42		3711.72	3014.60	
Seal #5	3549.74	3637.80	3404.14	3482.64	3474.96	3468.38	3458.12	2957.32	2992.48
	3530.32	3568.00	3530.86	3449.40	3396.46	3489.32	3462.24	2880.22	2889.10
	3554.56	3631.04	3542.96	3535.12	3473.20	3469.86	3486.02	2864.62	2830.20
	3718.04	3562.42	3494.42	3505.96	3511.04	3450.26	3373.26	2904.62	2926.14