

RESEARCH ARTICLE

Drivers of the dive response in pinnipeds; apnea, submergence or temperature?

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ABSTRACT

Long and deep dives in marine mammals are enabled by high mass-specific oxygen stores and the dive response, which reduces oxygen consumption in concert with increased peripheral vasoconstriction and a lowered heart rate during dives. Diving heart rates of pinnipeds are highly variable and modulated by many factors, such as breath holding (apnea), pressure, swimming activity, temperature and even cognitive control. However, the individual effects of these factors on diving heart rate are poorly understood because of the difficulty of parsing their relative contributions in diving pinnipeds. Here, we examined the effects of apnea and external sensory inputs as autonomic drivers of bradycardia. Specifically, we hypothesized that (1) water stimulation of facial receptors would – as is the case for terrestrial mammals – enhance the dive response, (2) increasing the facial area stimulated would lead to a more intense bradycardia, and (3) cold water would elicit a more pronounced bradycardia than warm water. Three harbor seals (*Phoca vitulina*) and a California sea lion (*Zalophus californianus*) were trained to breath hold in air and with their heads submerged in a basin with variable water level and temperature. We show that bradycardia occurs during apnea without immersion. We also demonstrate that bradycardia is strengthened by both increasing the area of facial submersion and colder water. Thus, we conclude that the initiation of the dive response in pinnipeds is more strongly related to breath holding than in terrestrial mammals, but the degree of the dive response is potentiated autonomically via stimulation of facial mechano- and thermo-receptors upon submergence.

KEY WORDS: Bradycardia, Breath hold, Facial receptors, Heart rate, Harbor seal, California sea lion

INTRODUCTION

Marine mammals must access two vital – but spatially separated – resources: food at depth and oxygen at the surface. Because of this spatial and hence temporal separation, individuals capable of performing long dives have the potential to reach more food patches and catch more prey per dive, thereby increasing fitness (Aguilar et al., 2008; Watwood et al., 2006; Wilson et al., 1993). This selection pressure has prompted a series of breath-hold adaptations

enabling extreme diving capabilities in marine mammals compared with terrestrial mammals (see seminal review by Ponganis, 2015). One primary adaptation that enables prolonged apnea is the dive response: a significant and selective peripheral vasoconstriction upon submersion that saves blood oxygen for the central hypoxia-intolerant organs such as the lungs, heart and brain (Irving et al., 1942; Panneton, 2013; Scholander et al., 1942), while allowing the oxygen partial pressure in the muscles to drop to levels that allow for oxygen release from myoglobin (Davis, 2014; Ponganis, 2011). The increase in vascular resistance caused by this vasoconstriction is matched by a proportional decrease in cardiac output via a diving bradycardia [heart rate (f_H) below resting values] (Elsner et al., 1964; Murdaugh et al., 1966; Ponganis et al., 1990). Thus, bradycardia is considered a good relative measure of the dive response (Hindle et al., 2010; Irving et al., 1941; Murdaugh et al., 1961; Thompson and Fedak, 1993).

All mammals studied have a dive response that can be initiated by apnea and facial submersion in water (Kooyman, 1989; Panneton, 2013; Ponganis, 2011). In marine mammals, this response is well developed and subject to modulation by several different drivers during diving. Upon facial submersion, thermo- and mechano-receptors are activated by water, triggering an increase in vagal tone (Elsner et al., 1966, 1964). When marine mammals dive, increasing pressure at depth apparently strengthens the dive response (Ferrigno et al., 1997; Hicks et al., 2004; Hindell et al., 1992; Kooyman and Campbell, 1972) through inactivation of pulmonary stretch receptors caused by compression of the lungs (Andersson and Schagatay, 1998; Drummond and Jones, 1979; Kooyman and Campbell, 1972). As the dive progresses, bradycardia, and therefore probably vasoconstriction, intensifies to maintain oxygen supply for the hypoxia-intolerant organs, while bradycardia decreases in the last part of the dive and during ascent (Andrews et al., 1997; Hindell et al., 1992; McDonald and Ponganis, 2013). Here and during prey capture events, diving bradycardia may be antagonized by increased muscular activity (e.g. movement during prey capture events or when returning to the surface) (Davis and Williams, 2012; Williams et al., 1991). This increase in activity may result in increased blood flow to the working muscles to maintain aerobic respiration, which is matched by an increase in f_H to maintain a stable blood pressure (Davis and Williams, 2012; Noren et al., 2012; Williams et al., 2015). Finally, in addition to physiological factors, the dive response of marine mammals seems to be subject to conscious expectations of dive duration (Elmegaard et al., 2016; Jobsis et al., 2001; Ridgway, 1975).

The dive response in marine mammals has traditionally been assumed to be triggered by apnea in combination with submersion, specifically water stimulation of facial thermo- and mechano-receptors, as observed in terrestrial mammals such as humans and dogs (Elsner et al., 1963; Gooden, 1994; James and Daly, 1972). This idea is based on classic studies of marine mammals showing

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pronounced bradycardia during forced submersions (Grinnell et al., 1942; Irving et al., 1942, 1941; Scholander et al., 1942). Notably, seals have been shown to exhibit marked bradycardia during asphyxiation – in the absence of submergence – induced by application of a wet towel to the face (Elsner et al., 1970). Furthermore, blocking the infraorbital branch of the trigeminal nerve during submersion revealed that the onset of bradycardia was delayed by suppressing stimulation of facial receptors (Harrison and Kooyman, 1968). Clearly, both apnea and water stimulation influence diving bradycardia; however, the extent to which these factors, or other factors such as temperature (Filingeri and Havenith, 2015; Schuitema and Holm, 1988) or cognitive control, contribute to the dive response remains unresolved (Irving et al., 1942; Jobsis et al., 2001; Scholander, 1963).

Additional clues concerning relative contributors to the dive response are provided by studies of seals and other pinnipeds measured under more voluntary conditions. Ridgway (1975) conditioned a California sea lion on land to exhibit an extreme bradycardia response on an acoustic command that was lower than the bradycardia exhibited by the same individual when conditioned to remain stationary underwater, highlighting the influence of cognitive control on heart rate in marine mammals. Outside the laboratory, wild elephant seals (*Mirounga angustirostris*) show pronounced decreases in f_H during spontaneous breath holds on land (Castellini et al., 1994; Falabella et al., 1999), similar to levels that occur during the initial phase of diving. This observation demonstrates the importance of apnea, both with and without submersion, and also suggests that cardiac responses under voluntary or relatively natural conditions may be different from those demonstrated under forced conditions.

Here, we sought to identify the relative contributions of several factors influencing diving bradycardia by training three harbor seals (*Phoca vitulina*) and a California sea lion (*Zalophus californianus*) to lower their head into a basin while resting on land, following the approach of Elsner (1965) and Elsner et al. (1964). Specifically, we recorded cardiac responses while the animals placed their heads into a basin with no water, or with varying levels of water and with different water temperatures, to determine (1) whether the dive response needs water to be initiated, (2) whether increasing the area of facial submergence results in a more pronounced bradycardia, and (3) the effect of head submersion in warm water versus cold water. We hypothesized that water stimulating the facial area combined with apnea would cause a deeper bradycardia compared with the same duration of apnea alone. Additionally, we hypothesized that increasing the facial area stimulated would result in a more pronounced dive response and that colder water would produce a greater dive response, as seen in terrestrial mammals (Panneton, 2013; Schuitema and Holm, 1988). The experimental approach enabled targeted drivers of cardiac responses to be examined in the absence of swimming activity and hydrostatic pressure, as well as confounding effects of forced submersion.

MATERIALS AND METHODS

Research facilities and animals

The experiments were conducted with three trained adult harbor seals (*Phoca vitulina* Linnaeus 1758; Family Phocidae): two males (identified as Svante and Sprouts) and one female (Naja); and one adult female California sea lion [*Zalophus californianus* (Lesson 1828); Family Otariidae] (Ronan). Naja and Svante were studied at the Fjord and Bælt Center in Kerteminde, Denmark, from September 2016 to June 2017. They were 17 and 18 years of age and weighed 70 and 79 kg, respectively. Sprouts and Ronan were

studied at Long Marine Laboratory in Santa Cruz, CA, USA, from March to June 2017. They were 29 and 9 years of age and weighed 108 and 72 kg, respectively. The four study subjects were long-term captive animals that were experienced in performing cooperative research tasks; aside from the advanced age of harbor seal Sprouts, none had health issues that would influence the cardiac or respiratory responses measured in this study. All animals were trained using operant conditioning and positive reinforcement. Desired behavioral responses were marked by a whistle (conditioned reinforcer) and a subsequent reward of freshly thawed herring, capelin or sprat fish. Animal diets were not constrained for experimental purposes.

Animal ethics

The data collected at Long Marine Laboratory were collected under authorization from the National Marine Fisheries Services of the United States under Marine Mammal Research Permit 18902, under the oversight of the Institutional Animal Care and Use Committee of the University of California, Santa Cruz. Data collection at the Fjord and Belt center was conducted under approval by the IACUC of Aarhus University.

ECG recording and initial processing

f_H was determined from ECG measurements collected using a custom-built monitoring system. The system consisted of (1) a flat wooden base with two embedded $20 \times 30 \times 0.3$ cm steel plates that acted as conductors for the electrical signal (ECG electrodes; Fig. 1A), (2) a GRASS P55 differential biopotential amplifier (Astro-Med® Inc., West Warwick, RI, USA) that filtered (1 Hz low pass, 10 kHz high pass) and amplified (10,000 times) the analog signal received from the ECG plates, (3) a National Instruments USB-6210 (Austin, TX, USA) data acquisition card that converted the conditioned analog signal to digital (sampling rate 12 kHz, 16 bit), and (4) a battery-powered laptop computer that also controlled the data acquisition card and stored the data via a custom-written LabView (National Instruments, Austin, TX, USA) virtual instrument (Fig. 1A).

The LabView software enabled additional filtering between 30 and 70 Hz to improve the quality of the heart beat signal (Fig. 1A). Additionally, the software allowed for keystroke annotations to be made in real time to the data file. For each trial, we noted animal movement and respiration, in addition to trainer commands.

The filtered ECG data were subsequently processed in MatLab v. R2016b (The MathWorks, Inc., Natick, MA, USA) using custom-written scripts, including a heart beat peak detector. All peak detections were manually checked for possible false detections or missed beats and errors were corrected. Instantaneous f_H was calculated as 60 s divided by the time difference between two neighboring heart beats. The f_H was assigned to the time of the second beat.

Trials were recorded with two HD HERO2 (GoPro Inc., San Mateo, CA, USA) cameras, allowing us to confirm annotations made on the data file. One HERO2 recorded the entire setup from the surface and the other one, placed at the bottom of a basin, focused on the seal's face. Video and f_H data files were synchronized by tapping a finger on the f_H monitor while simultaneously filming the event. The tapping event was easily recognized in the voltage output and clearly visible on the video.

To prevent movement artifacts from confounding f_H measures, data from periods with strong animal movements were excluded from analysis. Likewise, periods of low signal-to-noise ratio or signal dropouts were manually removed from the recorded ECG trials.

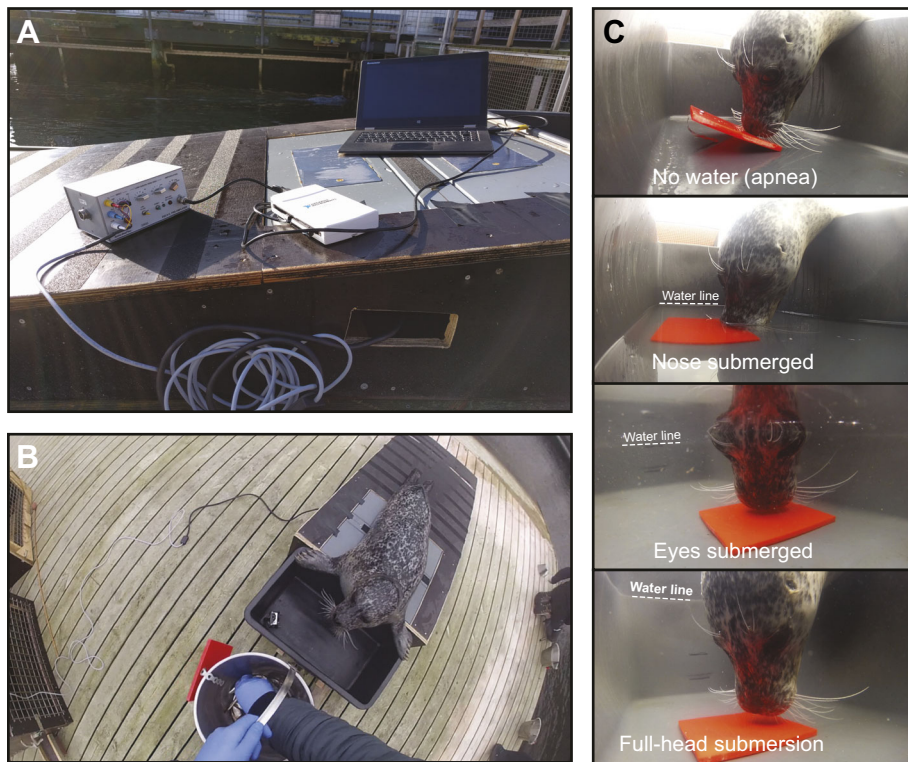


Fig. 1. Experimental setup. (A) Complete recording chain consisting of two ECG electrodes connected to a GRASS pre-amplifier (10,000 times amplification). A DAQ device (National Instruments USB-6210) converted the analog signal to digital and the data were saved as a text file using a custom-written LabView program on the laptop. (B) Animal position shown by a harbor seal lying on the ECG electrodes located on a ramp used to facilitate facial submersion in experiments 2, 3 and 4. The animals would place their head in the basin, which was filled with different levels of water. (C) Conditions in experiment 2; no water, nose submerged, eyes submerged and full-head submersion, covering the animal's face to above the ears. Full-head submersion was also the condition used in experiment 3 and 4.

Experimental setup and protocol

General method

To facilitate facial submersion, trials took place on land on a custom-made, slip-resistant ramp that lifted the animal's head ~30 cm above the ground. From the ramp, the seal could lower its head into a rectangular plastic basin with minimal physical effort (Fig. 1B). The wires from the ECG plate were protected by a rubber cover or hidden within the ramp to minimize noise in the ECG signal. Animals were trained to position their ventral surface on the ECG electrodes with their fore-flippers aligned with the top of the ramp (Fig. 1B) while remaining calm and relaxed; this minimized muscle/movement artifacts and encouraged normal breathing by the animal.

Experiment 1: baseline

To obtain data on respiratory sinus arrhythmias (RSA) during normal breathing for comparison with breath-hold f_H , we conducted a baseline trial for each animal. Each animal lay on the ECG plate for 10 min with minimal movement. Heart beats and respirations were recorded allowing investigation of f_H in periods of apnea and eupnea. In addition, both Sprouts and Ronan were rewarded for extended apneas while positioned on the ramp with their head out of the water and the longest breath hold from Sprouts and from Ronan are included as examples.

Experiment 2: effects of facial submersion

To test the hypotheses that (1) facial water stimulation triggers a dive response and (2) the degree of facial submersion influences the level of bradycardia, we measured f_H at different levels of facial submersion (Fig. 1). Before each session, the basin was filled (or not) with seawater from the animals' living enclosure and the animal was positioned on the ramp with the ECG plates. For each trial, the animal was cued by the trainer to lower its head into the basin, exposing him/her to one of four possible conditions: (i) no water in the basin, (ii) ~5 cm of water in the basin, just enough to cover the

nostrils, (iii) ~15 cm of water, just above their eyes, and (iv) full-head submersion, where the water covered the animals' ears (Fig. 1C). For each subject, 15 replicate trials were conducted for each treatment. Naja and Svante were tested with all four conditions, Sprouts and Ronan were tested with no water, nose submerged and full-head submersion. Svante and Naja finished all trials for one treatment before performing the next treatment in the following order: no water, nose submerged, eyes submerged and full-head submersion. To test for possible training effects, Naja and Svante were exposed to the no-water treatment a second time at the end of all the trials. In this second round of no-water treatment, only eight trials were conducted. Trial conditions for Sprouts and Ronan were randomized throughout testing to minimize the effect of training.

After the animal was positioned on the ramp, it rested for a minimum of 1 min before the start of each trial to ensure that the activity from moving onto the ramp did not influence the f_H . Each trial consisted of a 30 s pre-submersion period, a 30 s submersion, and a 30 s post-submersion period. The submersion interval started when the animal had its head below the edge of the basin (trials with no water) or at the point where it broke the water surface (trials with water). In both cases, the animal touched its nose to a plastic target affixed to the bottom of the basin until recalled by the trainer after exactly 30 s (Fig. 1C). During the no-water trials, the three harbor seals spontaneously held their breath during 'submersion'; however, the sea lion (Ronan) typically took a few breaths during the first few seconds of the submersion interval. Svante and Naja were occasionally fed during the start of the post-submersion interval and again following the conclusion of the post-submersion interval. Sprouts and Ronan were not fed over the interval from the start of the trial through to the end of the post-submersion period.

Experiment 3: effects of water temperature

To test whether water temperature influenced the observed dive response, two harbor seals, Naja and Svante, performed

additional trials. We used the same experimental set up as described for experiment 2, but with two basins placed next to each other rather than one. Prior to each session, one basin was filled with cold water (0–5°C) and the other basin with warm water (30–35°C). The typical ambient seawater in the seals' enclosure and during other experimental trials was 0–15°C and under seasonal influences.

During each session, each of the two seals performed four separate trials: two cold-water and two warm-water full-head submersions, alternating between the temperatures. As above, each trial comprised a 30 s pre-submersion period, a 30 s submersion period and a 30 s post-submersion period.

Experiment 4: effects of submersion duration

To examine whether the animals would reach their minimum f_H for a trained submersion during 30 s submersions, several long submersions were performed by the harbor seal Sprouts and the sea lion Ronan. The experimental setup was identical to the full-head submersion trials in experiment 2, but the subjects were cued to voluntarily dive for 3, 4 or 5 min. Sprouts performed two 3 min trials, two 4 min trials and one 5 min trial; Ronan performed six 3 min trials. Interspersed between Ronan's 3 min trials were a total of six additional 30 s submersion trials; these trials were conducted to measure the f_H response during a 30 s submersion when the sea lion would anticipate a 3 min submersion. Data collection for experiments 1 and 2 were completed before the animals were trained to perform long-duration dives in experiment 4.

Trial summary

A summary of trials is provided in Table 1. Each animal performed one 10 min trial of baseline f_H measurements while resting on the f_H sensor with their head out of water (experiment 1). Additionally, one extended apnea in this resting configuration, obtained opportunistically from Sprouts and Ronan, is included as an example. The response to facial submersion was examined in all four animals (experiment 2). Only Svante and Naja completed the trial conditions with their eyes submerged. In experiment 3, Naja and Svante performed 15 trials each of the alternating cold- and warm-water treatments. To examine the possible effects of conditioning, these seals performed eight additional no-water trials following completion of experiments 2 and 3. Finally, in experiment 4, Sprouts performed a series of long-duration dives (3–5 min) and Ronan performed six 3 min submersions and six 30 s submersions while anticipating a 3 min submersion.

Data processing

Because of the intrinsic bimodal nature of pinnipeds' f_H as a result of the RSA, a threshold between the low and the high levels of f_H was established for the baseline data collected in experiment 1. This allowed us to categorize each heart beat (and associated instantaneous f_H) as apneic or eupneic. All calculated f_H from each of the 10 min baseline trials were plotted in a histogram. Here, the bimodal distribution of f_H was clearly evident and therefore the sum of two Gaussian distributions was fitted to the histogram. The minimum fitted value between the two peaks was used as the threshold separating apneic or eupneic f_H and verified by video recordings that allowed detection of the subjected animal's respiration.

The f_H from the 30 s and 3 min submersion trials from experiments 2–4 were collapsed into 1 s bins synchronized to the start of the submersion. When the f_H was less than 60 beats min^{-1} , not every second could be assigned a f_H and thus that second was assigned to the following f_H . The mean f_H for each second was calculated from the binned f_H . The estimated threshold between eupnea and apnea f_H was used to divide pre- and post-submersion binned f_H of each second into a eupnea f_H or an apnea f_H . The mean f_H of each second for eupnea and apnea, respectively, was then calculated.

Statistics

Model 1: to test the effect of facial stimulation on f_H in the three harbor seals, we used a linear mixed model (LMM) with Gaussian error distribution and an identity link function. In this model, water level was the fixed effect and the random effects were: (1) time from the start of submersion, as each f_H was dependent on its preceding f_H , (2) individual animal, as f_H depended on each animal that conducted the trial, and (3) the trial number (between 1 and 15), as f_H could be influenced by training effects from successive trials.

Model 2: because Naja and Svante had one additional intermediate water treatment (eyes submerged) and a second round of the no-water condition, we performed a separate LMM (Gaussian error distribution, identity link function) with just the two seals to test for the effect of facial stimulation on f_H with water level as the fixed effect. Like model 1, this model adjusted for time, individual animal and trial number.

Model 3: to test the effect of facial stimulation on f_H in the California sea lion, Ronan, a LMM (Gaussian error distribution, identity link function) similar to model 1 was used; however, animal was not a random factor as there was only one subject.

Table 1. An overview of trials completed by three harbor seals (Naja, Svante, Sprouts) and one California sea lion (Ronan)

	Condition	Naja	Svante	Sprouts	Ronan
Experiment 1	Baseline 10 min rest on land	1	1	1	1
	Extended breath hold in air	–	–	1	1
Experiment 2 (30 s submersions)	No water (breath hold in air)	15	15	15	15
	Nose submerged	15	15	15	15
	Eyes submerged	15	15	–	–
	Head submerged	15	15	15	15
	2nd no water (effect of training)	8	8	–	–
Experiment 3 (30 s submersions)	Head submersion in cold water	15	15	–	–
	Head submersion in warm water	15	15	–	–
Experiment 4	30 s head submersion (anticipating 3 min submersion)	–	–	–	6
	3 min head submersion	–	–	2	6
	4 min head submersion	–	–	2	–
	5 min head submersion	–	–	1	–

Each row represents a different condition and experiment number; each cell shows the number of trials for each animal in each condition.

Model 4: to test for the effect of water temperature on f_H , we conducted a LMM (Gaussian error distribution, identity link function) with water temperature as the fixed effect, adjusted for the random effects of time, individual animal and trial number.

The first and last 2 s of every dive interval were excluded from all models to ensure the test conditions were fully achieved. The models were created with random intercepts and random slopes which resulted in better fits than with random intercepts alone; they

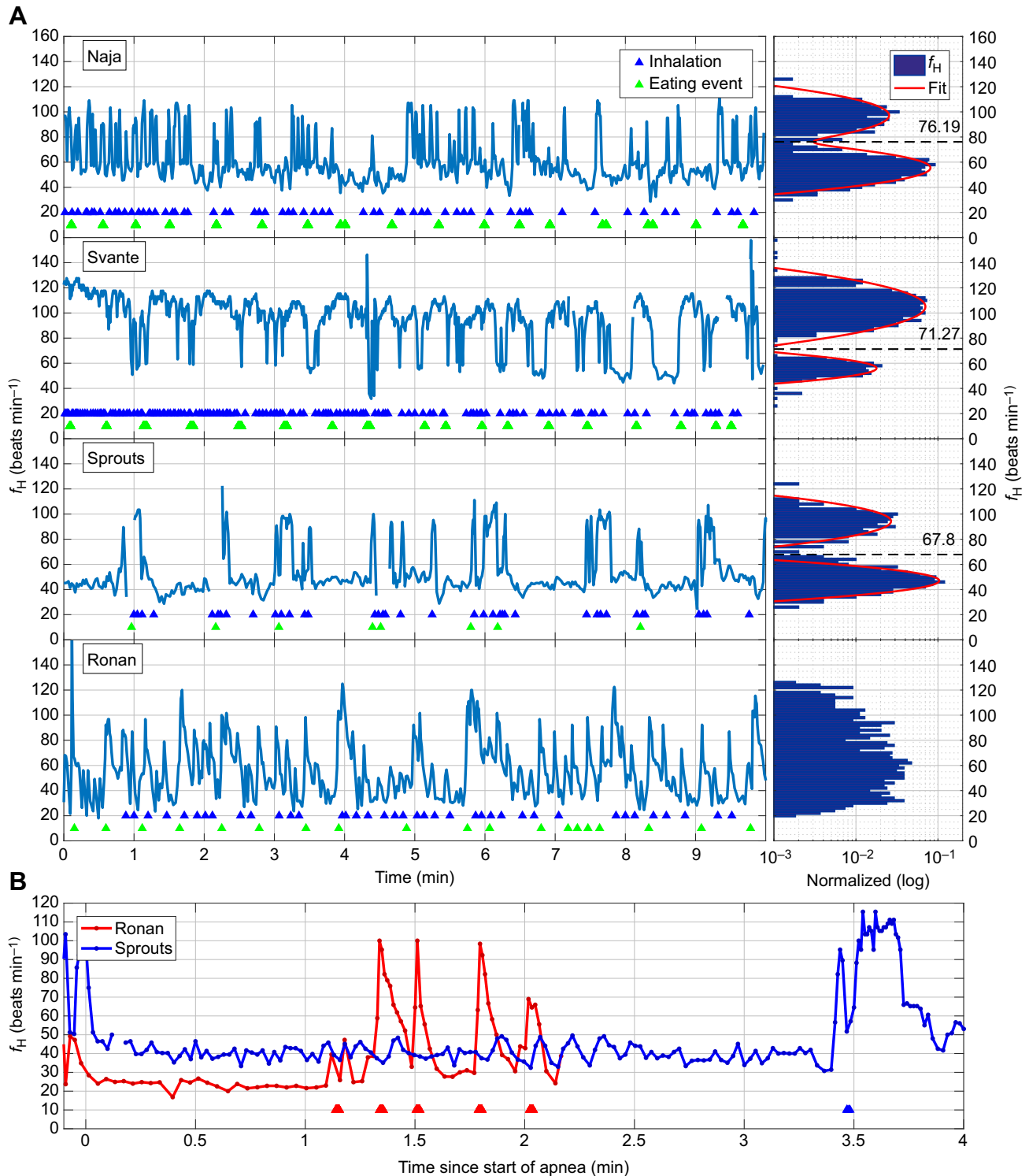


Fig. 2. Baseline and apnea trials on land without water. (A) Instantaneous heart rate (f_H) and ventilations during a 10 min resting trial on land conducted with three harbor seals (Naja, Svante and Sprouts) and a California sea lion (Ronan). Triangles mark inspirations (blue) and eating events (green). Panels to the right show the relative distribution of f_H binned into 1 s bins on a logarithmic scale for each animal. The dashed line and number mark the minimum fitted f_H between the two peaks of the fitted gaussian distribution. (B) f_H during maximum observed apnea duration at rest on land in air for Ronan and Sprouts. Time 0 marks the start of the apnea period and red (Ronan) and blue (Sprouts) triangles mark inspirations.

Table 2. Number of heart beats and instantaneous heart rate (f_H) measured during the 10 min rest in experiment 1 for three harbor seals (Naja, Svante, Sprouts) and a California sea lion (Ronan)

	Naja	Svante	Sprouts	Ronan
Total no. of beats	599	926	622	548
Overall mean f_H (beats min^{-1})	65.63 \pm 0.9	98.37 \pm 0.7	60.10 \pm 1.0	63.18 \pm 1.0
Threshold f_H (beats min^{-1})	76.19	71.27	67.80	—*
No. of beats: apnea	431 (72%)	106 (11%)	441 (71%)	—*
No. of beats: eupnea	168 (28%)	820 (89%)	181 (29%)	—*
Mean f_H : apnea (beats min^{-1})	53.4 \pm 0.3	53.6 \pm 0.6	46.2 \pm 0.3	—*
Mean f_H : eupnea (beats min^{-1})	96.9 \pm 1.1	104.1 \pm 0.4	94.0 \pm 1.0	—*

Data are reported as the number of heart beats in the total interval, during apnea and during eupnea, as well as the overall mean f_H , and the mean f_H during apnea and eupnea. The threshold f_H separated intervals of apnea and eupnea. Mean \pm s.e.m. values are stated and numbers in parentheses are the number of beats relative to the total number of beats for each animal.

*The California sea lion, Ronan, did not show a bimodal resting f_H and therefore there was no threshold determined between apnea and eupnea that could allow calculation of a mean apnea and eupnea f_H .

were run with the MatLab (R2016b) glmfit.m function, with a significance level of 0.05 in t -statistics. Residuals were evaluated and no assumptions were violated.

RESULTS

Experiment 1: baseline

All four animals displayed strong patterns in f_H associated with respiration (Fig. 2A). The harbor seals exhibited f_H alternating

between 35–60 beats min^{-1} during apnea and 80–120 beats min^{-1} during eupnea. The change in f_H was more variable in the sea lion as a result of multiple consecutive inspirations during eupnea, but there were still rapid, less-intense shifts in f_H in response to each of these ventilations (Fig. 2A). Respiration rates differed between the three harbor seals. Svante had the highest respiration rate with few and short apnea periods, resulting in a higher overall mean resting f_H compared with Naja and Sprouts (Table 2). In contrast, Sprouts

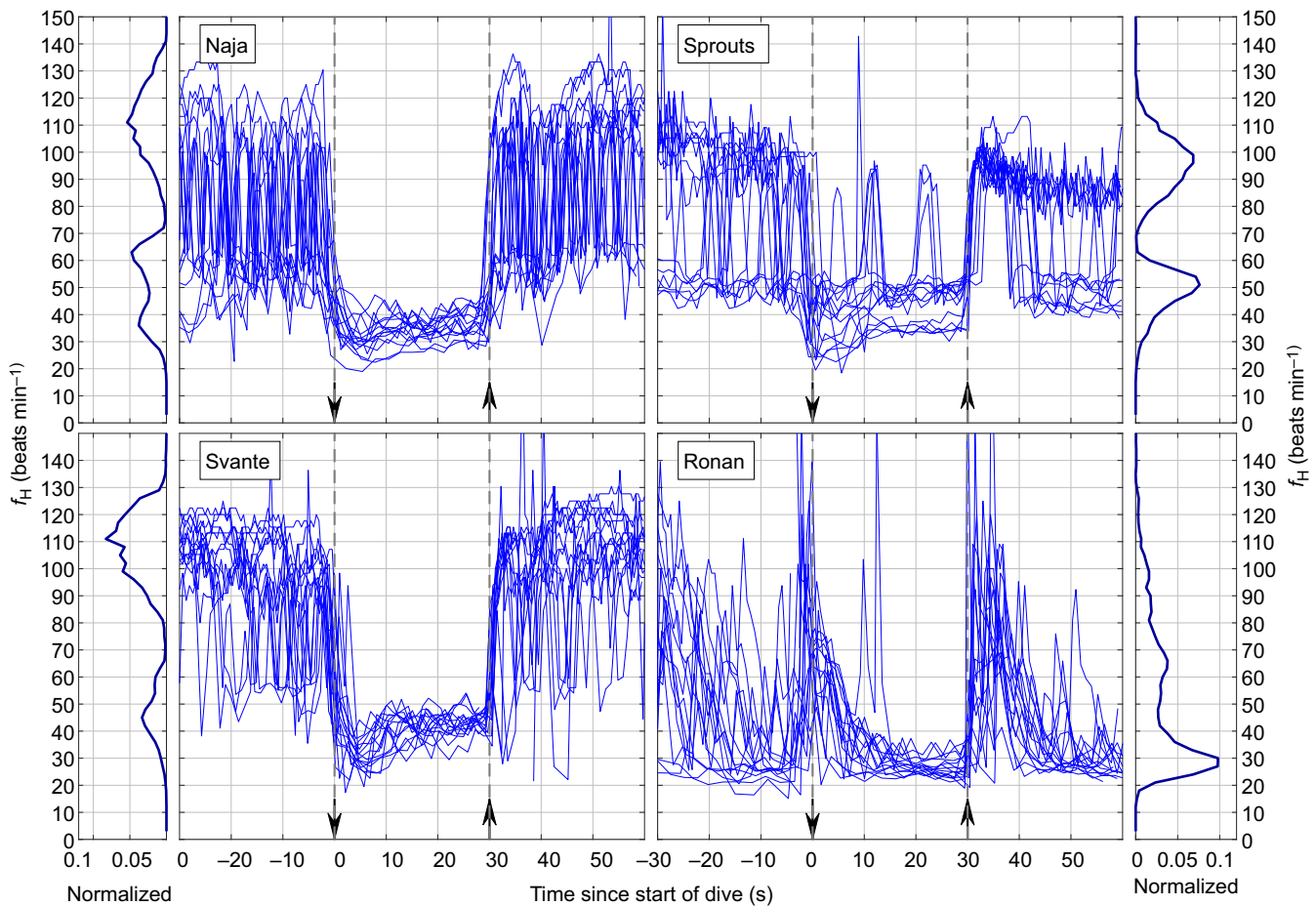


Fig. 3. Instantaneous f_H profiles from experiment 2 during full-head submersions in a basin with ambient water. Experiments were completed by three harbor seals (Naja, Svante and Sprouts) and one California sea lion (Ronan) while lying on an ECG electrode plate on land (15 trials each). Arrows mark the start (↓) and end (↑) of the submersion. The side panels show the sum of the normalized distribution of f_H for all full-head submersions for each animal, indicating a bimodal distribution in the three seals and a monomodal distribution in the sea lion.

had the lowest respiration rate, with long apnea periods with a low f_H . When resting and breathing normally, Naja, Svante and Sprouts had an obvious bimodal f_H distribution with a cluster of high f_H related to respiratory inspirations (eupnea) and a cluster of low f_H related to respiratory expirations and breath holds (apnea) (Fig. 2). Ronan, the Californian sea lion, had a more homogeneous distribution of f_H with an overall mean similar to that of Sprouts and Naja (Table 2), but with a minimum apnea f_H of 18 beats min^{-1} (Fig. 2A). During the longest extended breath hold without water,

Sprouts' f_H dropped to 30 beats min^{-1} over 3 min and 30 s and Ronan's to 17 beats min^{-1} (Fig. 2B) over 70 s. The thresholds for f_H between apnea and eupnea determined from the Gaussian distributions were similar for the three harbor seals (Table 2). Additionally, mean apnea and eupnea f_H were similar, within 11 beats min^{-1} for the three harbor seals (Table 2). No threshold, and thereby also no mean f_H during apnea and eupnea, could be determined for Ronan because of her rapidly shifting f_H between low and high extremes.

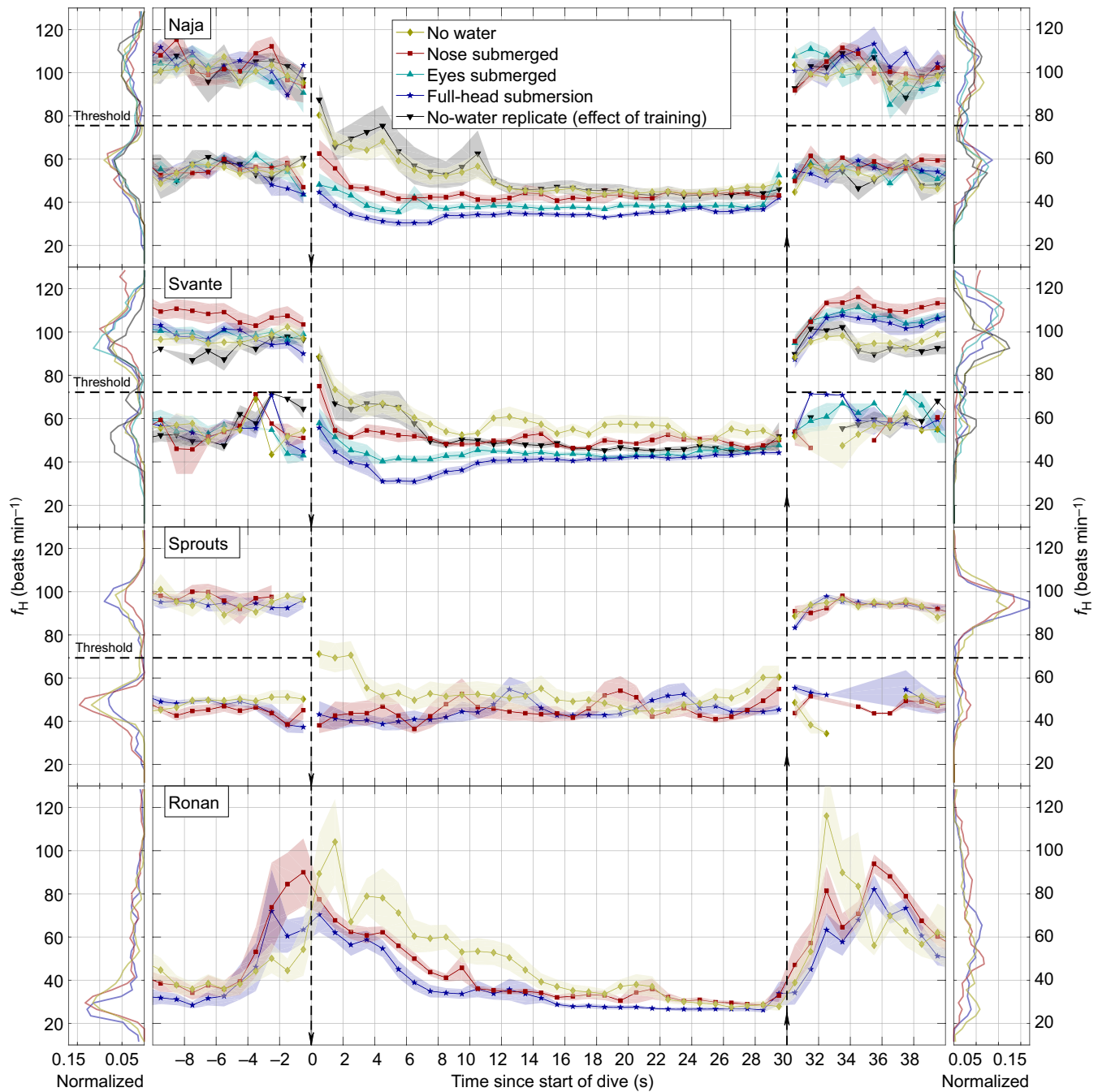


Fig. 4. Mean instantaneous f_H profiles during head submersion in a basin with different water levels. Three harbor seals (Naja, Svante, Sprouts) and a California sea lion (Ronan) were trained to lower their head into a basin with no water, or perform nose, eye or full-head submersion (15 trials of each condition except for 8 trials in the no-water replicate; data are means of each 1 s bin; shaded areas show the s.e.m.). Naja and Svante performed a second round of no-water trials after all trials were completed to test for a conditioning response. Sprouts' and Ronan's trials were randomized to prevent conditioning. Dashed lines with arrows mark the start (l) and end (t) of a dive. Side panels show normalized distribution of f_H for each treatment binned in 3 s intervals and plotted with a three-point running average.

Experiment 2: effect of facial submersion

To examine the effect of water on the dive response, the animals performed head submersions in a basin with different levels of water. A full-head submersion was expected to stimulate most facial thermo- and mechano-receptors, whereas submersion in an empty basin should not stimulate any facial receptors. Within the first 10 s of the 30 s full-head submersions, the f_H reached the lowest level in all three harbor seals, with a mean minimum f_H of 27.9 ± 1.1 beats min^{-1} (Naja), 27.5 ± 1.5 beats min^{-1} (Svante) and 29.9 ± 2.1 beats min^{-1} (Sprouts). This initial dip was followed by a stabilization of f_H at a level ~ 10 – 15 beats min^{-1} higher than the lowest level (Fig. 3). The two harbor seals Naja and Svante also showed this initial dip in trials of intermediate submersions (eyes submerged) but not in trials with no water or in trials where just their nose was submerged. Sprouts only showed the initial dip in full-head submersion trials (Fig. 4). In contrast to the harbor seals, Ronan showed a more gradual decline in f_H in the submersion trials (partial and full), with a mean minimum f_H of 24.0 ± 0.8 beats min^{-1} observed at the end of the 30 s full-head submersion. The steepest drop in f_H still occurred during the first 10 s of the dive (from 60 – 90 to 25 – 30 beats min^{-1}), after which f_H continued to decrease gradually (Figs 3 and 4). During submersion intervals with no water, Ronan's f_H initially increased before starting the gradual decline as a result of occasional breaths in the first few seconds of the on-target period (Fig. 4). The decrease in overall mean submersion f_H for all four animals was more pronounced when a larger facial area was stimulated with water, except between nose submerged and full-head submersion with

Sprouts (Fig. 5). The harbor seals Naja and Svante, tested with four conditions (model 2), showed a significant drop in f_H from trials with no water to nose submerged ($P < 0.01$), from nose submerged to eyes submerged ($P < 0.01$) and from eyes submerged to full-head submersion ($P < 0.01$). In the model with all three harbor seals (model 1), there was also a significant drop from no water to nose submerged ($P < 0.01$) and from nose submerged to full-head submersion ($P < 0.001$). The sea lion Ronan did not show a significant drop in f_H from the no-water to nose-submerged conditions ($P > 0.05$); however, she exhibited a drop in f_H from nose-submerged to full-head submersion trials ($P < 0.05$).

To test for possible effects of conditioning, trials with no water were repeated with Naja and Svante following data collection for experiment 2. During this second round of no-water trials, the f_H for Svante and Naja combined was not lower than that during the initial trials with no water ($P > 0.05$), and neither was f_H significantly higher than in the nose-submerged trials ($P > 0.05$). However, these trials still showed a higher f_H than those in which the eyes were submerged ($P < 0.01$).

Experiment 3: effects of temperature

To test the hypothesis that colder water stimulating facial receptors elicits a more pronounced bradycardia, Naja and Svante performed 30 s head submersions into both cold and warm water. Cold water elicited a deeper bradycardia than warm water in the seals ($P < 0.001$) (Fig. 5). Naja and Svante had a mean minimum f_H of 32.7 ± 1.8 and 23.0 ± 0.9 beats min^{-1} in warm water, and 26.3 ± 2.8 and 18.4 ± 1.5 beats min^{-1} in cold water, respectively. The greatest

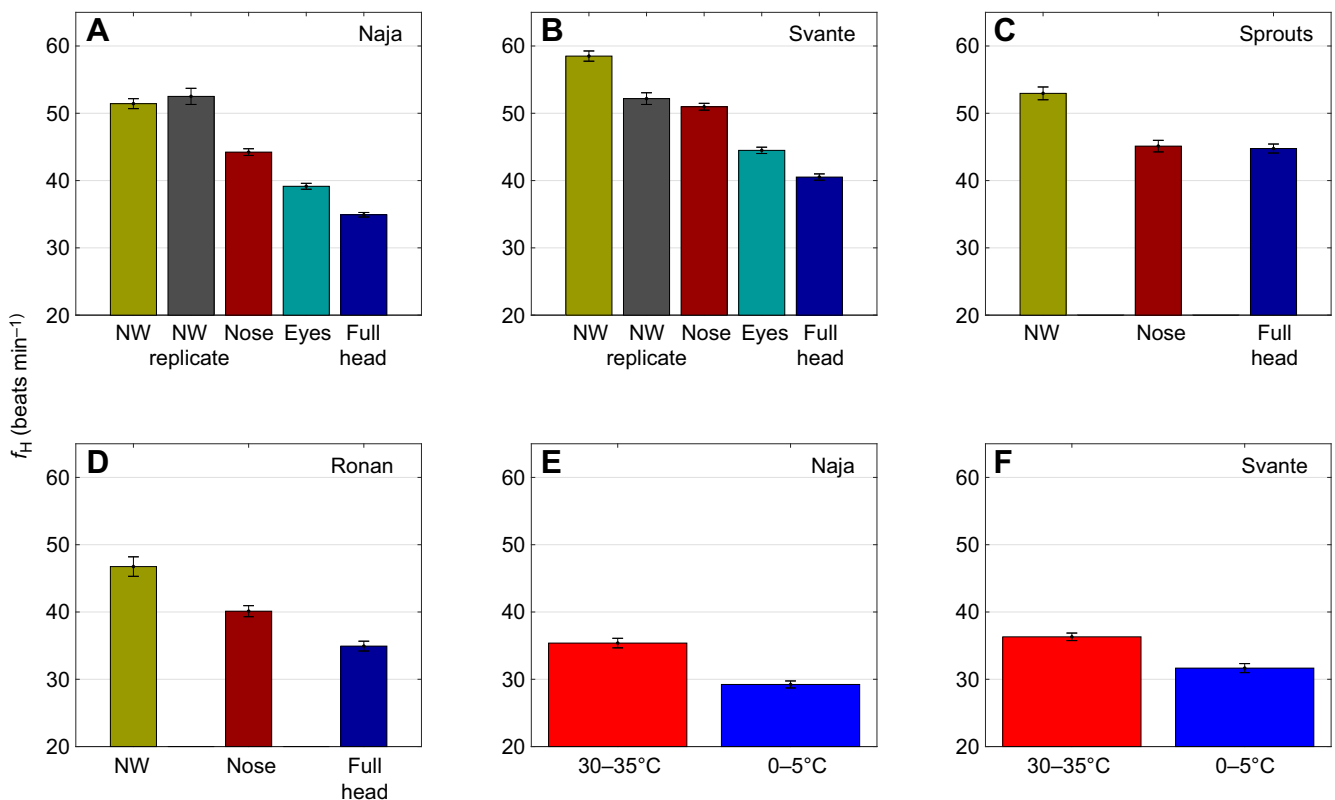


Fig. 5. Mean f_H during 30 s submersion trials with different levels of water and temperature. (A,B) The two harbor seals, Naja and Svante, exposed to all conditions in experiment 2. No-water (NW) replicates were conducted after the seals had been exposed to all other treatments to test for any effect of physical training. (C,D) The results from one harbor seal, Sprouts (C), and from one California sea lion, Ronan (D), both exposed to three different water conditions in experiment 2. (E,F) The effect of water temperature on full-head submersions in cold (0 – 5°C) and warm water (30 – 35°C), for the two harbor seals Naja and Svante in experiment 3. All animals were exposed to the same treatment 15 times except for the no-water replicate (8 trials only). Data are means \pm s.e.m.

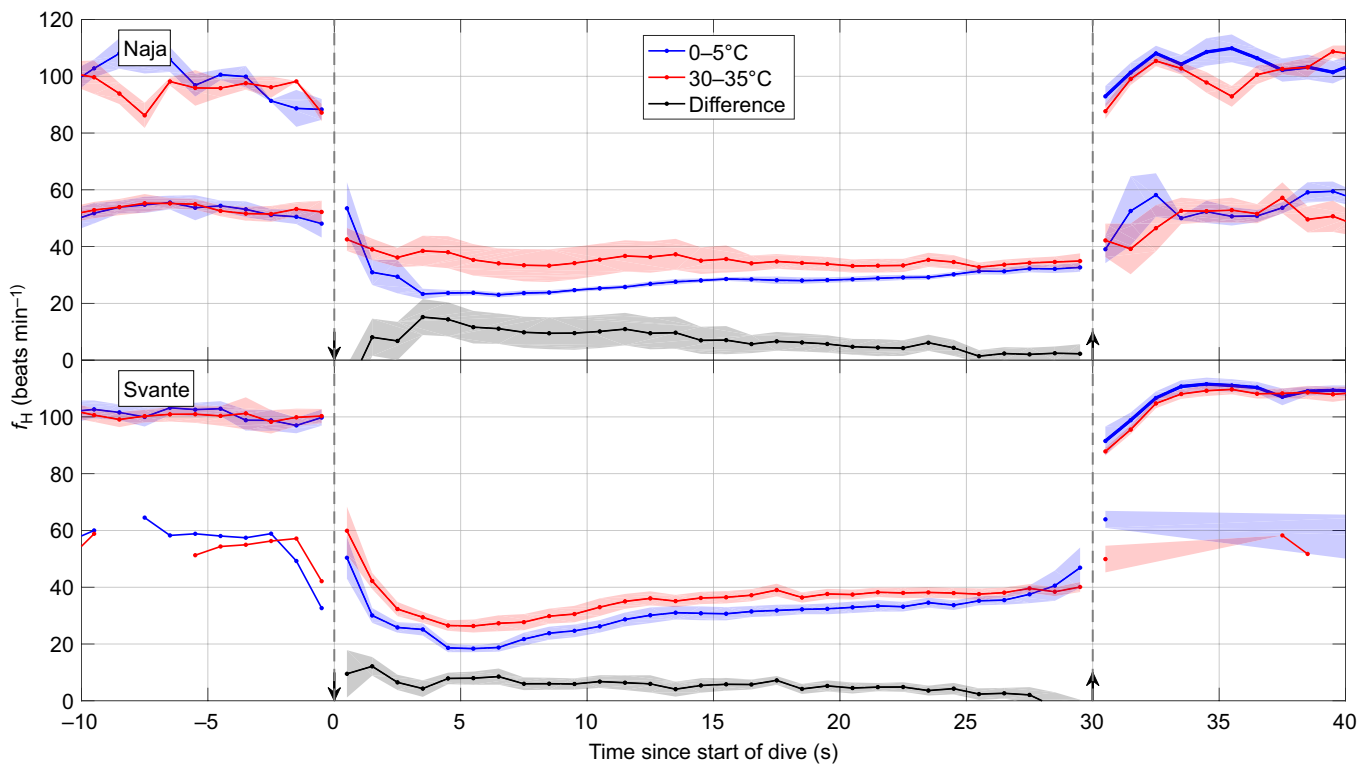


Fig. 6. Mean instantaneous f_H of two harbor seals, Naja and Svante, tested in experiment 3 during head submersion in cold and warm water. Means were calculated for each 1 s bin and shaded areas represent s.e.m. The black line is the difference in f_H between the two treatments. In the 10 s pre- and post-dive periods, the f_H is divided into high and low f_H from the animal's estimated eupnea/apnea threshold. If no shaded area is plotted, the number of f_H measurements in that second is <2.

difference between the cold- and warm-water treatment for Naja occurred 3–5 s into the dive; however, the f_H in the cold-water treatment gradually increased during the remainder of the dive, ending at similar values to those observed in the warm-water treatment (Fig. 6). In contrast, the difference in f_H for Svante was stable throughout the entire dive (Fig. 6). Both seals showed a decrease in overall mean submersion f_H from warm- to cold-water conditions (Fig. 5).

Experiment 4: effects of long dives

To examine whether stronger bradycardia responses would occur during prolonged submersion, Ronan and Sprouts performed extended submersion trials following experiment 2. During the 3 min full-head submersions, Ronan showed a deeper bradycardia in the first 30 s compared with that in the 30 s full-head submersions performed earlier (experiment 2). However, there was no difference in f_H response between the 3 min head submersions and the 30 s

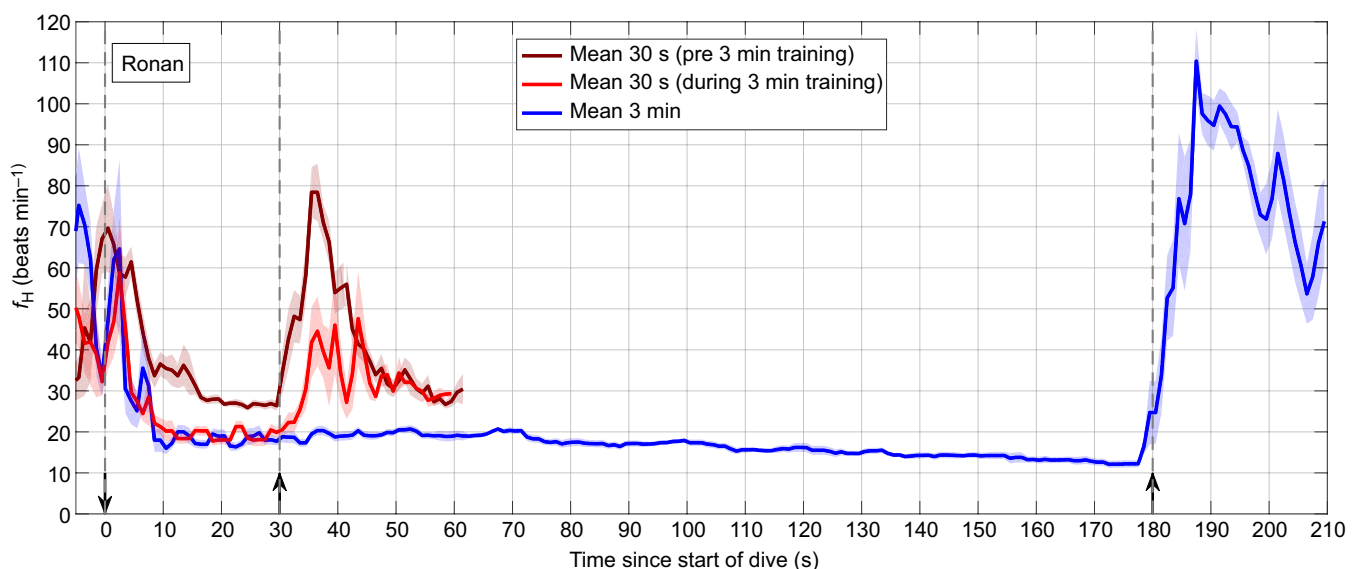


Fig. 7. Full-head submersions (3 min or 30 s) of a California sea lion (Ronan) conducted in experiment 4. f_H was lower during 30 s head submersions following 3 min head submersion training (light red) compared with f_H pre-training (dark red). Shaded areas represent s.e.m.

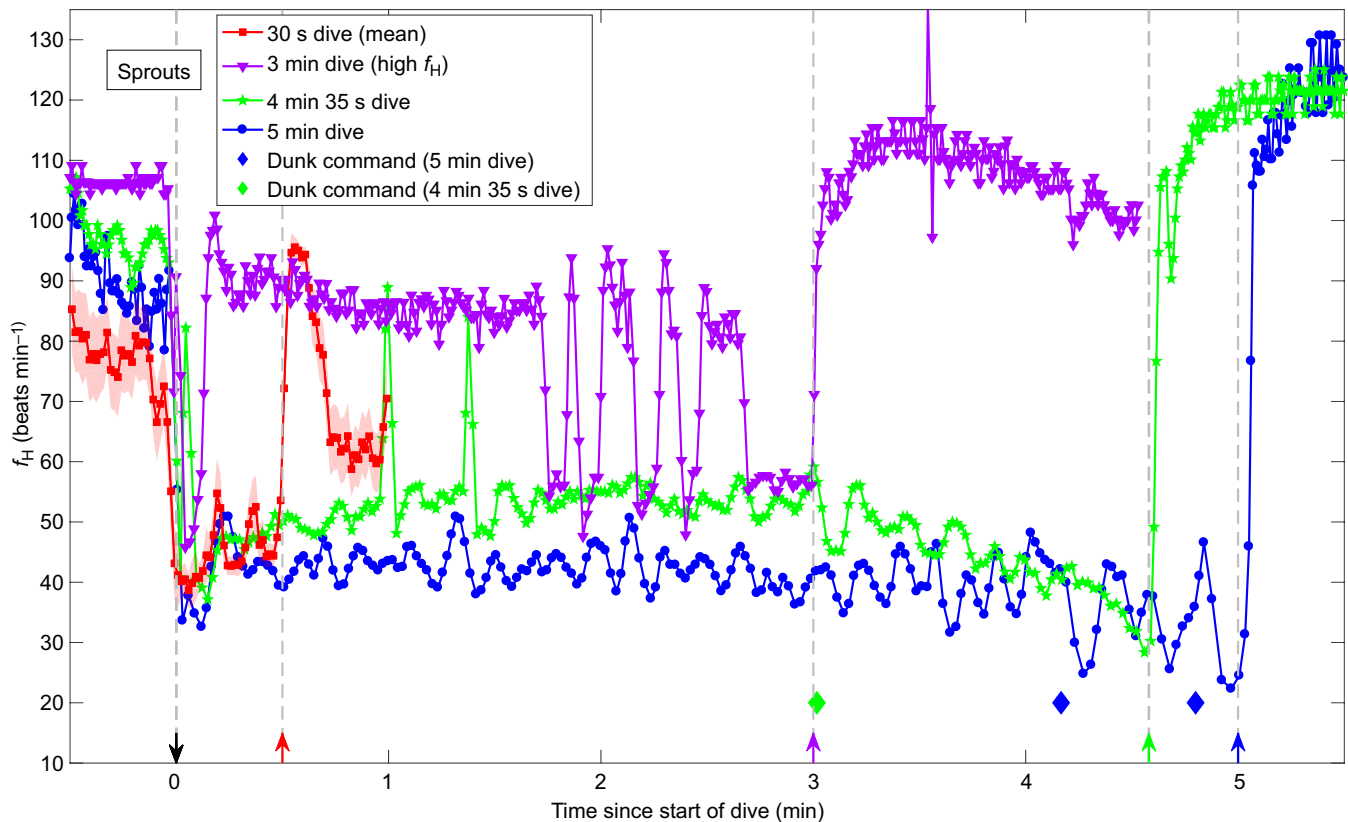


Fig. 8. Instantaneous f_H profiles for one harbor seal (Sprouts) performing long-duration dives in experiment 4. Diamonds mark when extra verbal prompts (dunk command) were given to encourage the seal to continue the behavior. Arrows mark the start (black) and end (colored) of each dive. The 30 s plot (red) is the mean of 15 trials and the red shaded area represents s.e.m.

head submersions performed during the 3 min submersion training period (Fig. 7) when Ronan was anticipating 3 min submersions. The mean minimum f_H was 24.0 ± 0.8 beats min^{-1} during 30 s full-head submersion trials from experiment 2, 16.9 ± 1.0 beats min^{-1} during 30 s full-head submersions in the 3 min submersion training period, 15.0 ± 1.1 beats min^{-1} in the initial 30 s of the 3 min submersions, and, finally, 11.7 ± 0.7 beats min^{-1} in the full 3 min submersions. The harbor seal Sprouts also performed long-duration head submersions of 3, 4 and 5 min. Only during submersions longer than 3 min did Sprouts' f_H decrease to values below the stable f_H established in the first 10 s of the submersion. The lowest f_H value of 22 beats min^{-1} was measured at the end of the longest head submersion (Fig. 8). During two submersions, a verbal signal was given to prompt Sprouts to remain submerged, and at these points, f_H dropped instantly after the command (Fig. 8). During one 3 min full-head submersion, the initial decrease in f_H lasted for 10 s followed by an increase in f_H to a level between 80 and 100 beats min^{-1} . After 2 min, f_H became more variable, finishing at a level of 55–60 beats min^{-1} (Fig. 8; high f_H).

DISCUSSION

In terrestrial mammals, the dive response is initiated by water stimulation of facial thermo- and mechano-receptors, which activates a dive reflex through the trigeminal nerve (Andersen, 1963; Gooden, 1994; Gooden et al., 1976; Panneton et al., 2010). This reflex occurs independent of breath holding, although it is more pronounced in combination with apnea (Brick, 1966). Apnea alone in humans only induces weak bradycardia (Hong et al., 1967; Olsen et al., 1962), an effect that is greatly intensified during submergence (Andersson

et al., 2000; Kawakami et al., 1967). In the case of marine mammals, it is less clear what actually constitutes a dive response, as most studies of the dive response have occurred in actively swimming animals or animals under severe stress, and strong bradycardia can occur during apnea independent of submergence (Castellini et al., 1994; Falabella et al., 1999). This diverse group of champion divers, with large oxygen stores and cardiovascular oxygen-saving responses, are well adapted to protracted periods of apnea (Davis, 2014). Furthermore, the intermittent breathing pattern of pinnipeds, in combination with these intensive cardiovascular responses to apnea, results in strong RSA as a function of eupnea and apnea even when resting on land (Castellini et al., 1994; Lapierre et al., 2004; Lin et al., 1972; McDonald and Ponganis, 2014). Thus, is the notion of a strong bradycardia in diving mammals mainly a result of tachycardia during eupnea to ensure high lung perfusion rates during periodic ventilation?

Indeed, our three trained harbor seals and one trained California sea lion all exhibited large RSA while resting on land (Fig. 2A), similar to what has been observed in these species previously (Lin et al., 1972; McDonald and Ponganis, 2014; Päsche and Krog, 1980). Such a strong RSA confirms that resting pinnipeds alternate between intervals of tachycardia when breathing and bradycardia between breaths while resting on land. The depth of bradycardia exhibited during apneustic periods of intermittent breathing is the primary contributor to overall diving bradycardia. During a 3.5 min breath hold in air, the harbor seal Sprouts had a minimum f_H of 30 beats min^{-1} compared with his mean low f_H of 46 beats min^{-1} during rest in air. Thus, harbor seals occasionally exhibit a bradycardia during prolonged apneas in air that are comparable to

those observed during prolonged stationary dives (Fig. 8). In turn, the f_H during short (3 s) full-head submersion dives in water was higher than that exhibited during prolonged apnea in air. Similar observations of an equally strong bradycardia in air and during submersions have also been made during free-ranging dives within the aerobic dive limit in California sea lions (McDonald and Ponganis, 2014), elephant seals (Castellini et al., 1994) and emperor penguins (Meir et al., 2008). We could not compute a meaningful mean low RSA for the sea lion, Ronan, because of her rapidly changing f_H at rest during brief apneas, which are characteristic of sea lions (Lin et al., 1972). However, her lowest RSA at rest was similar to that during prolonged apnea in air and the initial 30 s of the 3 min full-head submersions, which were all lower than the RSA for 30 s full-head submersion during experiment 2. Thus, protracted apneas while the animals are breath holding in air on land display all the features of a diving response. These observations highlight the difficulty in defining what qualifies as a dive response in breath-holding mammals – a problem that is further compounded by the fact that exercise-modulated tachycardia in marine mammals can lead to quite high f_H during unconstrained dives (Williams et al., 2015).

If pronounced bradycardia is present in the absence of submersion, it raises the question of whether the marine mammal dive response, contrary to that of terrestrial mammals, is decoupled from water stimulation of facial receptors (Gooden, 1994; James and Daly, 1972; Schuitema and Holm, 1988). We examined whether bradycardia in trained pinnipeds is potentiated by a reflex response to submersion or whether bradycardia is caused only by apnea and/or by other drivers such as cognitive or volitional control. By conditioning the animals to lower their head into a basin while on land (Elsner, 1965; Elsner et al., 1964), we were able to isolate the effect of facial water stimulation from other variables such as temperature, pressure and activity that a freely diving animal would experience. As hypothesized, measured f_H during submersion was significantly lower than that during apnea alone. Additionally, for both California sea lions and harbor seals, we showed that the larger the facial area stimulated, the more pronounced the bradycardia observed, which is probably a consequence of the size of the receptor field stimulated. The two harbor seals Naja and Svante strengthened their apnea bradycardia in air from the start to the end of the experimental period during additional training. However, the strengthened bradycardia from these repetitive trials cannot explain why the observed bradycardia during apnea in air at the end of the experimental period was less pronounced than the bradycardia observed during the initial treatment with water. It is therefore unlikely that the effects of training can explain the full bradycardia response to submersion in these seals. Although comparison of Ronan's f_H response was precluded because she breathed during no-water trials, there was still a difference between nose-submerged trials and full-head submersion trials for the sea lion. Thus, facial thermo- and mechano-receptors do indeed potentiate the dive response in pinnipeds.

Given these results, we suggest that water stimulation of facial receptors contributes to the dive response, but less so than in terrestrial mammals, including humans (Gooden, 1994). In fact, the dive response in humans is strongest in cold water and even more pronounced with large differences in temperature between the air and water (Kawakami et al., 1967; Schagatay and Holm, 1996). The same relationship was found in the two harbor seals diving voluntarily in the present study, which also showed the most pronounced dive response in cold water. Interestingly, this potentiation of the dive response by cold water was not found by Dykes (1974) during forced submersions where the diving response had perhaps reached a maximum through fear.

Although water stimulation intensified the dive response in these pinnipeds, the dive responses for the different water treatments started to merge after the initial reduction in f_H , and the effect of water immersion tapered off during the experimental trials. This indicates that the physiological response to water is phasic, similar to that of common cold-sensing thermo-receptors (Campero and Bostock, 2010). This phasic response was also observed during long-duration submersions performed by the harbor seal Sprouts. During one 3 min dive with full facial submersion, this seal showed an elevated f_H of around 80–95 beats min^{-1} . Despite this rather high f_H , Sprouts still showed an initial strong bradycardia. As the experimental setup kept the different drivers constant, this initial dip in f_H can be attributed to water stimulation, providing evidence that water triggers a phasic reflex that potentiates the dive response upon face immersion in the harbor seal via stronger parasympathetic tone. In contrast, the sea lion Ronan did not exhibit a dip in f_H during the initial phase of extended dives, which suggests a weaker reflex to facial submersion in this individual. We speculate that this lack of an initial dip in f_H could be a consequence of strong cognitive modulation.

Studies on free-ranging sea lions have shown variable levels of initial bradycardia based upon apparent expectations of dive durations (McDonald and Ponganis, 2014). The ability to modulate the dive response exclusively by the anticipation of dive duration has recently been shown for harbor porpoises (*Phocoena phocoena*), in which possible drivers of the dive response were controlled (Elmegaard et al., 2016). Similarly, our findings suggest that the California sea lion employed cognitive control to modulate the dive response: initial f_H (immediately following the start of submersion) for Ronan was lower after 3 min submersion training, suggesting an anticipatory response to prolonged submersion. This notion is also supported by an equally pronounced bradycardia in response to 30 s submersions performed unexpectedly between 3 min submersions, implying anticipation of a 3 min submersion.

Even stronger evidence for cognitive control is provided by the harbor seal Sprouts. During long-duration submersions, this seal appeared to be on the verge of ending the dive prematurely in a few trials. When that happened, the trainer prompted the seal with a verbal signal to remain submerged, which led to an immediate drop in his f_H . This response was similar to that reported by Jobsis et al. (2001) in a restrained harbor seal habituated to 3 min forced-submersion dives. After 2 weeks of daily 3 min dive trials, a dive was unexpectedly extended to 5 min. After 3 min of submersion, f_H dropped immediately, concurrent with a strong decrease in muscle blood flow (Jobsis et al., 2001). As other possible drivers such as activity, temperature and pressure remained constant during the extended dives in our study and in that of Jobsis et al. (2001), the sudden changes in cardiovascular response can be attributed to the seal recognizing that it has to hold its breath for longer than anticipated, leading to more conservative oxygen management. In contrast to an early study by Irving et al. (1942) – where similar cardiovascular responses were observed when harbor seals were exposed to shouting, clapping and pinching – Sprouts' response was not caused by fear. In the present training paradigm, the seal was always able to lift his head from the basin and breathe on his own initiative; there was no consequence other than delaying reinforcement. Additionally, at least in one extended dive, the seal showed an elevated f_H , probably due to anticipation of a shorter duration submersion. Because all physical variables remained constant following the initial response to diving, cognitive/volitional control is, in our view, the likely driver influencing f_H on these extended-duration dives. The ability to cognitively control the dive response may explain why Dykes (1974) did not observe

any effect of different water temperatures during forced submersions. The consequences of severe distress leading to maximum bradycardia may have made it impossible to discern the effect of temperature during forced submersions.

From the examples and data discussed, it seems likely that both harbor seals and California sea lions modulate their dive response to some degree through assessment and decision making, consistent with early claims of cognitive control in pinnipeds (Kooyman and Campbell, 1972; Murdaugh et al., 1961; Ridgway, 1975). Whether foraging pinnipeds optimize their dive response to match their expectations of the duration and depth of the dive or anticipated food patch quality will be an interesting research topic in the future.

Conclusion

Here, we showed that three harbor seals and a California sea lion display pronounced bradycardia during spontaneous apneic periods on land that can be just as strong as those during diving without active swimming or pressure changes, indicating that a large portion of their dive response is, in fact, a response to apnea alone. Furthermore, we showed that water stimulation of facial thermo- and mechano-receptors results in more rapid and intense bradycardia. The larger the facial area stimulated and the colder the water, the more pronounced the observed dive bradycardia. This cardiac response was strongest immediately following submersion, leading us to propose that the initiation of bradycardia is facilitated by facial receptors with a phasic response. In the harbor seals, facial submersion potentiated initial bradycardia even when diving bradycardia was weak throughout the remaining dive. However, after the initial facial response, diving bradycardia seems to be modulated by cognitive or volitional control. This view is supported by changes in f_H during submersion in the seals and sea lion attributable to anticipatory responses. We therefore conclude that the main driver of the dive response in pinnipeds is apnea, the effects of which are potentiated by submersion and modulated by cognitive or volitional control.

Acknowledgements

The authors gratefully acknowledge the skills and dedication of J. Sills and the team at the Pinniped Cognition and Sensory Systems Laboratory at the Long Marine Laboratory, Santa Cruz, CA, USA. We thank J. S. Jensen and N. U. Kristiansen for help with developing the ECG equipment and K. Beedholm for developing the custom-made ECG-recording LabView virtual instrument. We also thank two anonymous reviewers for providing very helpful comments that improved the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.K., C.R., J.L.S., P.T.M.; Methodology: J.K., J.H.K., J.L., F.J., P.T.M.; Software: J.K.; Validation: B.I.M., P.T.M.; Formal analysis: J.K.; Investigation: J.K., C.R., J.L., J.L.S., P.T.M.; Resources: C.R., J.H.K., F.J., P.T.M.; Data curation: J.K., C.R.; Writing - original draft: J.K.; Writing - review & editing: J.K., C.R., B.I.M., P.T.M.; Visualization: J.K.; Supervision: P.T.M.; Project administration: J.K., J.H.K., P.T.M.; Funding acquisition: P.T.M.

Funding

This study was funded by the Danmarks Grundforskningsfond via frame grants to P.T.M.

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