

Activity not submergence explains diving heart rates of captive loggerhead turtles

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Summary: Heart rates of undisturbed loggerhead turtles were not different whether turtles were submerged or out of water. Rather, heart rates changes were driven by turtles' activity level.

Abstract

Marine turtles spend their life at sea and can rest on the seafloor for hours. As air-breathers, marine turtles' breath-hold capacity is a function of oxygen (O₂) stores, O₂ consumption during dives, and hypoxia tolerance. However, some physiological adaptations to diving observed in mammals are absent in marine turtles. This study examines cardiovascular responses in loggerhead turtles, which have even fewer adaptations to diving than other marine turtles, but can dive for extended durations. Heart rates (f_H) of eight undisturbed loggerhead turtles in shallow tanks were measured using self-contained ECG data loggers under five conditions: spontaneous dives, resting motionless on the tank bottom, resting in shallow water with their head out of water, feeding on squid, and swimming at the surface between dives. There was no significant difference between resting f_H while resting on the bottom of the tank, diving, or resting in shallow water with their head out of water. Heart rate rose as soon as turtles began to move and was highest between dives when turtles were swimming at the surface. These results

suggest cardiovascular responses in captive loggerhead turtles are driven by activity and apneic f_H is not reduced by submergence under these conditions.

Introduction

Loggerhead turtles can remain submerged at sea for as long as seven to eight hours (Hawkes et al., 2007; Hochscheid et al., 2007). While not as extraordinary as the initial reports in the 1970s of marine turtles hibernating on the ocean floor for months (Carr et al., 1980; Felger et al., 1976), these are notable durations considering, unlike freshwater turtles, marine turtles do not have significant extrapulmonary respiration capacity (Lutz and Bentley, 1985). Thus, as in marine mammals, marine turtles' breath-hold capacity is a function of the amount of stored oxygen (O_2), how fast they consume O_2 during dives, and how tolerant they are to low O_2 levels. However, some of the key physiological adaptations observed in diving mammals are not present or unknown in marine turtles. Loggerhead turtles in particular represent a unique model to study cardiovascular responses in a diving reptile because, despite their long dive durations, they have even fewer adaptations to diving than other marine turtles.

First, unlike marine mammals, which cut off muscle blood flow during forced submersions (Scholander, 1940), muscle blood flow appears to occur in forcibly submerged loggerhead turtles (Lutz and Bentley, 1985). An intense peripheral vasoconstriction during forced submersion was initially described in seals (Scholander, 1940), the archetypal diving mammal, and considered a mechanism to limit O_2 uptake by tissues and to defend central arterial pressure in the face of a severe reduction in heart rate (f_H). Peripheral vasoconstriction was sufficient to result in the cessation of muscle perfusion, which was demonstrated by the simultaneous build up of muscle lactate content and the lack of blood lactate accumulation during the forced submersion, and, then, the subsequent large increase in blood lactate after the seal was allowed to breathe again (Scholander, 1940). In green turtles, blood lactate remained low during the initial 30 to 60 minutes of a forced submersion, but then increased in the late stages of the submersion, suggesting peripheral vasoconstriction occurred initially, but could not be maintained for the entire submersion. In contrast, blood lactate accumulated throughout forced submersions of loggerhead turtles, implying continuous muscle perfusion in this species (Lutz and Bentley, 1985).

Second, while O₂ stores are elevated in marine mammals and, to a lesser degree, leatherback turtles, other marine turtles do not have increased O₂ stores (Lutcavage et al., 1990; Lutcavage et al., 1992; Ponganis, 2015). In many homeothermic divers, myoglobin (Mb) concentrations are up to fifteen times higher than terrestrial animals, providing a significant O₂ store in muscle (Butler and Jones, 1997). The Mb-O₂ store can support maintenance of aerobic metabolism if muscle blood flow is limited during diving (Scholander, 1940; Williams et al., 2011). However, Mb concentrations in the muscle of loggerhead turtles are not elevated above terrestrial animals (Lutz and Bentley, 1985).

Although reduced f_H is the hallmark of the dive response, few studies have examined cardiac responses to diving in marine turtles. The classic dive response includes a rapid drop in f_H as the dive begins and low f_H during the dive until the ascent when f_H increases to near surface levels (Butler and Jones, 1997; Ponganis, 2015). The reduction in f_H in diving mammals is variable: ranging from minimal in manatees and moderate in the majority of dives of many pinnipeds and dolphins to severe (equivalent to forced submersion) in long dives of some animals (Andrews et al., 1997; Gallivan et al., 1986; McDonald and Ponganis, 2014; Thompson and Fedak, 1993; Williams et al., 2017; Wright et al., 2014). The one study on marine turtles diving at sea described only mild f_H reduction in diving leatherback turtles relative to surface f_H (Southwood et al., 1999).

The high cost of endothermy at least partially necessitates the extreme mechanisms required for marine mammals to remain submerged for extended durations. However, with marine turtles' low metabolic rate and hypoxic tolerance (including of the brain) (Berkson, 1966; Lutz et al., 1980; Lutz and Bentley, 1985), cardiac adjustments to reduce O₂ consumption during dives may not be as critical to their aquatic lifestyle. Studies on freshwater turtles suggest f_H changes are related to activity level and not whether turtles are submerged or on land (Krosniunas and Hicks, 2003). With few studies on f_H in marine turtles, the effect of activity level and submergence on f_H is unknown.

During summer, sub-adult and adult-sized loggerhead turtles forage in the near shore areas of the Sanriku Coast of Japan (Narazaki et al., 2015). On occasion, they are incidentally caught in set

nets by local fishermen. We took advantage of this seasonal incidental capture of loggerhead turtles to investigate their cardiac responses to voluntary dives. Our goals were to describe f_H patterns and investigate three questions about f_H of loggerhead turtles. First, is f_H during dives reduced relative to (1) surface f_H and (2) f_H when resting in shallow water with their head out of water? We predicted f_H at the surface would be higher due to the costs of ventilation and increased activity during surface intervals. However, with the apparent persistence of muscle blood flow during forced submersions, low Mb concentrations and the shallow depth of the experimental tanks, we predicted that turtles would not exhibit a significant decrease in f_H during dives versus resting out of water. Second, is f_H influenced by underwater activity of turtles? Again, because of apparent persistence of muscle blood flow during forced submersions, we predicted that the more turtles moved, the higher f_H would be in order to meet O_2 demands during activity. Finally, does f_H of turtles decrease as dive duration increases? We predicted that, because of their low metabolic rate and tolerance to hypoxia, turtles would not need to further reduce f_H in longer dives and that total heart beats would be closely correlated with dive duration. We attached time depth recorders (TDRs) and self-contained physiological data loggers to record f_H and behavior. Turtles swam, rested and foraged in large outdoor tanks during the experimental period and then were released back to the ocean after experiments were completed.

Materials and Methods

Animals & study site. Eight loggerhead turtles, *Caretta caretta* (Linnaeus 1758), were incidentally caught in or around Otsuchi Bay, Japan and transported to the University of Tokyo marine station (International Coastal Research Center, 39°21'05N, 141° 56' 04E) in the summers of 2013 and 2015. The sex of the eight turtles (58.5 ± 18.9 kg) was not determined. During the experiments, turtles were kept in outdoor, individual seawater tanks (6m x 3m x 1m) with continuous flow.

Instrument configurations. Each turtle was outfitted with a physiological data logger (Model: UUB/3-EPTb, UFI, Morro Bay, CA, USA) enclosed in a custom-made plastic waterproof housing (6.3 x 3 x 2 cm, 99g in air) as previously described (Williams and Hicks, 2016). The data loggers continuously recorded ECG at 50 Hz. A time depth recorder (TDR, 2013 - DT or D2GT, Little Leonardo, Tokyo, Japan, 16g in air, 1s sampling interval; 2015 - DST-Pitch and

Roll, Star-Oddi, Gardabaer, Iceland; 4 x 1.3 cm, 9 g in air; 1s sampling interval, resolution: +/- 0.6 cm; <0.1°C) was attached to the turtle's head with 5-min epoxy (Loctite Quick Set Epoxy, Henkel Corporation, Düsseldorf, Germany).

ECG implant. After mask induction of the turtle with 2-5% isoflurane in 100% O₂, anesthesia was maintained at 1-2.5% isoflurane with spontaneous ventilation. Respiration was continuously monitored. In 2013, ECG wires, sterilized with Nolvasan Solution (Zoetis, Parsippany, NJ, USA), were implanted percutaneously in the first two turtles, with one electrode implanted in the neck near the left edge of the carapace and the other in the skin below the carapace and above the right rear flipper. To improve the ECG signal for the last two turtles in 2013, one electrode was implanted in the skin of the neck as above and the other electrode was guided through a two millimeter diameter hole drilled through the carapace near the right rear flipper. The carapace was cleaned with a topical povidone iodine solution before and after drilling and the hole was treated with 2-3ml of 2% lidocaine as soon as it was drilled. All percutaneous implants were secured with sutures and tissue glue (3M; Vet-Bond, St Paul, MN, USA) and electrodes placed through the carapace were secured with tissue glue and holes were sealed with epoxy. Due to the high level of noise in all ECG signals in 2013, an intravascular bipolar pacing catheter (Model D97120F5, Edwards Life Sciences, Irvine, CA, USA), modified to record ECG signals, was used in all four loggerhead turtles in 2015. ECG catheters were inserted 24 to 32 cm percutaneously in the jugular vein through a peel-away catheter (5.5Fr or 6Fr, Cook Medical, Bloomington, IN, USA) (Meir et al., 2009; Ponganis et al., 2007) using ultrasound (MicroMaxx Ultrasound System, SonoSite Inc., Bothell, WA, USA) visualization as a guide to placement (Di Bello et al., 2010).

Instrument Attachment. A piece of ¼" square polyester mesh (1006, Delta knotless netting, Memphis Net and Twine, Memphis, TN, USA) approximately 8 cm x 5 cm with a 1 cm² Velcro™ patch in the center was secured to the upper midline of the carapace with 5-min epoxy. Data loggers were attached to the netting with a Velcro™ patch on the bottom of the housing and with plastic cable ties. With this attachment method, loggers could be removed and replaced easily. ECG catheters were connected to the data logger through underwater connectors (HUMG2-BCR & CCP, Sea-Con Brantner & Associates, Inc., El Cajon, CA, USA) and all

cables or wires were secured to the carapace using Tesa tape (tesa SE, Beiersdorf AG, Hamburg, Germany).

Diving Protocol. After recovery overnight, turtles were left alone in the tanks, free to dive, swim or rest. Turtle behavior was monitored with a TDR and visually from a third story stairwell in a nearby building. In some cases, turtle behavior was also videotaped. In 2015, turtles were fed dead squid several times a week.

Removal. Loggers and TDRs were taken off at the end of each experiment and data downloaded. All netting, epoxy and tape were removed from the carapace. Implanted sensors were taken out and skin incisions were closed with tissue glue. The holes through the carapace were treated with topical povidone iodine solution and then resealed with epoxy. After several days, skin incisions were no longer visible and holes through the carapace had begun to heal. These holes heal completely within 4 weeks (Southwood et al., 2003). At the end of all experiments, turtles were released into Otsuchi Bay. All procedures were performed in accordance with the guidelines of the Animal Ethics Committee of the University of Tokyo, and the protocol of the study was approved by this committee (Permit Nos. P13-17 and P15-16).

Data analysis. ECG data were plotted in a graphics program (Origin 2015-18, OriginLab Corporation, Northampton, MA) and a custom peak detection script (K. Ponganis, unpublished) was used to detect and mark QRS complexes in the ECG profiles. The intervals between marked R waves were used to calculate instantaneous f_H (Meir et al., 2008). To ensure accuracy, all marked R waves were visually confirmed.

An aerobic dive limit (ADL) is the duration beyond which lactate begins to accumulate above resting values, however, as ADLs are difficult to measure since they require a post-dive blood sample, a calculated aerobic dive limit (cADL) can be used to assess dive durations (Kooyman et al., 1983; Kooyman et al., 1980). In this study, a range of cADLs (O_2 stores/ O_2 consumption rates) were determined for each turtle based on previously estimated mass specific O_2 stores ($22.2 \text{ ml } O_2 \text{ kg}^{-1}$), and a previously measured O_2 consumption rate for North Pacific loggerhead

turtles corrected for water temperature and for body mass with a metabolic scaling factor (Kinoshita et al., 2018; Lutz and Bentley, 1985; Prange and Jackson, 1976). In this equation,

$$\dot{V}_{O_2} = 0.1098 \exp(0.0581T_w + 0.0075A), \quad (1)$$

T_w is the water temperature in Celsius, A is the percentage of time turtle was active and units are expressed as $\text{ml O}_2 \text{ min}^{-1} \text{ kg}^{-0.83}$. The cADL range was calculated for activity levels of the turtles from 100% to 0% (Kinoshita et al., 2018).

Conditions measured. Heart rate was determined during five conditions, which were associated with different turtle behaviors and activity levels. The dive condition encompassed the time between a turtle's last breath prior to putting its head underwater and re-surfacing. Dives were identified based on DST pitch and roll logger profiles and visual observations. The pitch and roll profiles were used to identify when turtles submerged or surfaced and when they were motionless at the bottom of tanks, but were not used to quantify activity levels within conditions. A dive was defined as having a minimum duration of two minutes and all shorter submergences were excluded from analyses. Post-dive surface intervals ("PSI") were defined as the time periods between dives when turtles were at the surface and did not include any short (< two min) submergences. Since 92% of PSIs were 15 minutes or less, PSIs longer than 30 minutes were also excluded from analyses. In 2015, feeding events were defined as the time turtles spent underwater either moving toward the squid or consuming squid and were identified from behavioral observations, TDR profiles and video recordings. Dives with feeding events were not included in any analysis of dives. One feeding event was analyzed per turtle. Two types of 10-minute resting periods were examined for each 2015 turtle: lying in a tank filled with water that reached the bottom of their carapace, but shallow enough so their head remained out of water ("shallow-water rest") and submerged and motionless on the bottom of the tank ("bottom rest"). For shallow water rest, we confirmed that each turtle's nares did not reach the water. Further, to minimize potential disturbance during shallow-water rest, turtles were not visually observed and DST pitch and roll profiles were examined to select these periods. As a result, breathing was not recorded and turtles could be apneic or eupneic during shallow-water rest. Bottom rest periods occurred during dives and these periods were included as part of dives within the dive condition.

Analysis. ECG data were successfully obtained in four turtles in 2013 and four turtles in 2015. To obtain f_H , duration and water temperature of different conditions or events, instantaneous f_H and TDR profiles (time, depth and water temperature) were plotted over a single 24-hour period in Origin for each turtle. The number and duration of dives, maximum duration per turtle, PSI duration and mean dive water temperature were determined during the 24-hour period for all eight turtles. Because R waves were not clearly discernible during movement in 2013 but were easily identified in 2015, only 2015 turtles were used for most f_H analyses. However, minimum instantaneous diving f_H was determined for the eight turtles as ECG signals were evident even in 2013 turtles when turtles rested on the bottom of the tank (Fig. S1A). For the 2015 turtles, feeding duration, total heart beats per dive and diving f_H (total heart beats divided by duration) were calculated. In addition, in 2015 turtles, the mean f_H and range of f_H were determined for all (1) dives, (2) PSIs, (3) feeding events, (4) bottom rest periods, and (5) shallow-water rest periods.

Statistics. We used linear mixed effect models (packages: lme4, lmerTest and psycho (Bates et al., 2015; Kuznetsova et al., 2017; Makowski, 2018; Pinheiro et al., 2018) implemented in R (version 3.2.3 (R Core Team)) to analyze the data. In all models, individual turtles were a random effect to account for repeated sampling. Water temperature was also included in all initial models as a fixed effect because it has a known effect on f_H and duration (Bentivegna et al., 2003; Southwood et al., 2003). All models were fit by maximum likelihood using Akaike Information Criterion (AIC). Models were compared using Chi-squared distributed likelihood ratios. P-values were calculated using Satterthwaite's method to estimate denominator degrees of freedom for t-statistics (Kuznetsova et al., 2017). Marginal and conditional r^2 values were obtained to determine goodness of fit (Nakagawa and Schielzeth, 2013). Residuals were checked to confirm model requirements, including normality and homoscedasticity, for all models. Heart rates were compared under the five activity conditions (dive, bottom rest, shallow-water rest, feeding, and PSI). Differences between conditions were compared with Tukey's post-hoc pairwise comparison. To assess changes in f_H in longer dives and because the calculation of diving f_H includes duration (total heart beats divided by dive duration), we evaluated the total number of heart beats as a function of dive duration and water temperature in 2015 turtles. We also analyzed the minimum instantaneous f_H in relation to dive duration in all turtles. To

investigate recovery after dives, we compared PSI mean f_H and diving f_H in 2015 turtles. Finally, we plotted PSI duration with dive duration in all turtles. Means are expressed as \pm s.d.

Results

Behavior. Deployments ranged from two to eight days. During the 24-hour observation period, turtles typically submerged and rested on the bottom of the tank for up to 53 minutes (Table 1). Dives per turtle during the 24-hour periods ranged from 29 to 58 for a total 328 dives (Table 1). Over 40% of dives were longer than 30 minutes and almost 60% were longer than 20 minutes (Fig. 1). Calculated ADLs ranged between 51 and 58 minutes for turtles at rest (Table 1). Mean and maximum dive durations for each turtle are reported in Table 1. Maximum dive durations were not followed by consistently high PSI durations, however, there was an evident increase in the minimum PSI duration in longer dives (Fig. 2).

During shallow-water rest, turtles were left alone in a tank with water that reached the bottom of their carapace, but shallow enough so their head remained out of water. Although turtles were primarily motionless during both dive and shallow-water rest, there was some intermittent activity. Dives included movement to and from the surface and shallow-water rest included some turtles moving, including head movements potentially during breathing, although breathing was not quantified. The lack of movement during bottom rest periods was confirmed from the pitch and roll profiles of turtles lying on the bottom of tanks. When turtles were fed squid, they slowly swam underwater toward the squid and paused briefly as they consumed the squid. Feeding was the most active underwater condition as turtles moved almost continuously. During PSIs, turtles swam continuously at the surface back and forth the length of tanks, intermittently lifting their head to breathe. Turtles did not float at the surface or have periods of inactivity during PSIs.

Diving f_H was lower than surface f_H , but not different from shallow-water rest f_H . Heart rates during diving, bottom rest, shallow-water rest, feeding and PSIs are reported in Table 2. In the model comparing f_H under five conditions, water temperature did not have a significant effect and was removed in the final, best fit model. Condition had a significant effect on f_H (Fig. 3, (F=18.3, DF=4, residual DF=12, $p<0.001$) and condition explained 77.4% of variation under the

model, while effect of individual contributed 2.5% of explanatory power ($R^2_m = 77.4$; $R^2_c = 79.9$). A post-hoc pairwise comparison showed PSI f_H was significantly higher than diving f_H ($p < 0.001$, $t = 6.8$, $df = 14$). However, there were no significant differences in f_H between shallow-water rest and dive ($p = 0.18$, $t = 2.4$, $df = 12$, Fig. 3) or shallow-water rest and bottom rest ($p = 0.12$, $t = 2.7$, $df = 14$, Fig. 3).

Heart rate increased as underwater activity levels increased. When turtles moved underwater, f_H increased by two- to three-fold and occasionally reached PSI f_H (Fig. 4, Table 2). Feeding f_H was significantly higher than bottom rest f_H ($p < 0.05$, $t = 3.5$, $df = 12$) and diving f_H ($p < 0.05$, $t = 3.2$, $df = 12$), but not shallow-water rest f_H ($p = 0.9$, $t = 0.81$, $df = 12$).

Heart rate relation to dive duration. The total number of heart beats was driven by dive duration and water temperature ($p < 0.001$, $X^2 = 476.3$, $df = 4$, $R^2_m = 0.87$, $R^2_c = 0.97$, Fig. 5A) as total beats increased by 5.4 and 15.0 beats for each additional minute submerged and degree Celsius increase in water temperature, respectively (duration: $p < 0.001$, $F = 373.2$, $df = 1$, residual $df = 2.8$; water temperature: $p < 0.001$, $F = 62.5$, $df = 1$, residual $df = 158.4$). Mean diving f_H versus duration is plotted in Fig. 5B for illustration. Mean minimum instantaneous f_H was significantly related to dive duration and water temperature ($p < 0.001$, $X^2 = 187.3$, $df = 5$, $R^2_m = 0.30$, $R^2_c = 0.77$, Fig. 6). The best predictive model for mean minimum instantaneous diving f_H included dive duration ($p < 0.05$, $F = 7.1$, $df = 1$, residual $df = 20.5$), water temperature ($p < 0.05$, $F = 38.9$, $df = 1$, residual $df = 95.4$) and an interaction between duration and water temperature ($p < 0.01$, $F = 12.1$, $df = 1$, residual $df = 20.7$).

Heart rate patterns. When turtles began to descend during dives, f_H dropped quickly from a range of 12 to 20 beats min^{-1} down to less than 10 beats min^{-1} (Fig. 4). While resting on the bottom, mean minimum instantaneous f_H was less than 6 beats min^{-1} for all turtles (Table 1). There was no significant difference between bottom rest f_H and diving f_H ($p = 1.0$, $t = 0.15$, $df = 14$, Fig. 3). After turtles returned to the surface, f_H was always the highest f_H as turtles swam continuously during the PSI (Table 2, Fig. 3). In addition to being higher than diving f_H , PSI f_H was significantly higher than bottom rest f_H ($p < 0.001$, $t = 6.9$, $df = 14$), shallow-water rest f_H ($p < 0.01$, $t = 3.2$, $df = 14$) and feeding f_H ($p < 0.05$, $t = 3.6$, $df = 14$). Dive duration and water temperature were significantly related to instantaneous mean PSI f_H ($X^2 = 91.3$, $p < 0.001$, $df = 4$, $R^2_m = 0.34$, $n = 4$, Fig 7), such that for each

h additional minute submerged, post-dive f_H increased by 0.13 beats min^{-1} ($p < 0.05$, $F = 13.3$, $df = 1$, residual $df = 3.6$). An increase in water temperature also had a significant effect on PSI f_H , raising f_H by 1.7 beats min^{-1} per degree Celsius ($p < 0.001$, $F = 37.9$, $df = 1$, residual $df = 156.6$).

Effect of temperature. Mean water temperature was slightly higher in 2013 (23.0 ± 0.4 °C) than 2015 (20.0 ± 0.3 °C) and varied among individual turtles (Table 1). Water temperature was not significant in all models, likely because it did not vary widely, especially among 2015 turtles (Table 1).

Discussion

The most important findings in this study are that (a) diving f_H and bottom rest f_H were not significantly different from f_H , when turtles were resting with their head out of water, (b) changes in f_H were largely driven by activity, and (c) f_H was not further reduced in longer dives.

Diving f_H was lower than PSI f_H , but not different from shallow-water rest f_H . While it is often assumed that all air-breathing vertebrates have a diving bradycardia (e.g., (Andersen, 1966)), the question of whether turtles experience a diving bradycardia is complicated by the control f_H to which the diving f_H is compared. If a diving bradycardia is defined as a lower f_H during diving compared to at the surface, then loggerhead turtles experienced a diving bradycardia under the conditions of the present study. Diving f_H was less than half the PSI f_H . In marine turtles freely diving at sea, leatherback turtles' diving f_H (17 beats min^{-1}) were only moderately lower than post-dive surface f_H (25 beats min^{-1}) (Southwood et al., 1999). However, it is difficult to compare these results because, in the present study, turtles were primarily resting on the bottom during dives, while leatherbacks were diving at sea and, thus, potentially more active (Southwood et al., 1999). While freely diving at sea, loggerhead turtles may also have higher f_H ; however, to date there have been no studies examining diving f_H in loggerhead turtles at sea.

On the other hand, if a diving bradycardia is defined as a significantly lower f_H during dives compared to a f_H at the same activity level either at the surface or on land (Stephenson et al., 1986), then loggerhead turtles did not experience a diving bradycardia. There was no significant difference in f_H of these turtles either during the entire dive or during rest in the bottom phase of

the dive compared to f_H at rest in shallow water with the turtle's head out of the water. While this study will not resolve how to define a diving bradycardia in marine turtles, at a minimum, these results suggest that f_H is not further reduced by being submerged. These results are consistent with the lack of difference in resting f_H , whether red-eared sliders were on land or underwater (Krosniunas and Hicks, 2003). There was also no difference in f_H between walking on land, underwater swimming or diving in red-eared sliders (Krosniunas and Hicks, 2003).

The comparison between shallow-water rest and bottom rest or diving f_H s is also complicated by the cost of ventilation during shallow-water rest. Although breathing was not monitored during shallow-water rest, the pitch and roll profiles indicated that, in some instances, breathing may have occurred. Ventilation in marine turtles requires use of respiratory muscles to expand lungs and neck muscles to raise the head, both of which should come at an energetic cost. However, O_2 consumption in resting loggerhead turtles did not increase during ventilation (Lutz et al., 1989). Similarly, estimates of the oxidative costs of breathing in freshwater turtles suggest it is low (from 1% of resting metabolic rate) (Jackson et al., 1991). The lack of a significant difference between shallow-water rest f_H and diving f_H or bottom rest f_H supports the low metabolic costs of ventilation in marine turtles or that our turtles were primarily apneic during the shallow-water rest periods.

Heart rate increased as underwater activity level increased. Changes in the f_H of submerged loggerhead turtles were driven by activity, similar to terrestrial animals as part of the exercise response. When turtles were motionless on the bottom of tanks, f_H was lowest at 6.3 beats min^{-1} . However, with any discernible movement, f_H increased (Fig. 4), at times nearing PSI f_H levels. The highest underwater f_H occurred as turtles swam underwater during feeding; it was significantly higher than bottom rest f_H and diving f_H . While diving at sea, the most vigorous underwater swimming occurs as turtles begin to descend, when stroke frequency and amplitude are highest (Hays et al., 2007). Our findings suggest f_H may be highest during the descent phase of dives. This activity-related increase in f_H also occurs in some diving endotherms. A number of marine mammals and birds have a graded dive response in which exercise appears to decrease the level of bradycardia (i.e., increase f_H) in some, but not necessarily all dives (Butler and Jones, 1997; Davis and Williams, 2012; Hindle et al., 2010; Noren et al., 2012; Signore and Jones,

1996; Williams et al., 2015). For example, in Steller sea lions, f_H was related to activity levels during shallow dives, but not deep dives (Hindle et al., 2010). Nonetheless, despite these varied responses, f_H typically remained below surface or resting levels in most dives of these other species.

However, this effect of underwater activity on turtle f_H is in stark contrast to other marine mammals and birds, including emperor penguins diving at sea. During the bottom phases of emperor penguins' deepest dives at sea, stroke rate was the highest (Williams et al., 2012). Yet, Wright et al. found during these deepest segments, emperor penguin f_H was at its lowest (Wright et al., 2014). Similarly, during the initial post-release dives of narwhals, f_H was maintained near ten beats min^{-1} , independent of stroke activity (Williams et al., 2017). By uncoupling workload from f_H , these divers conserve O_2 for hypoxia sensitive organs (the heart and brain). This contrast between turtles and diving endotherms on the effect of activity was also observed in forced submersions of marine turtles and seals, where struggling elicited a significant increase in f_H in green turtles, but not in seals (Berkson, 1966; Scholander, 1940).

Like marine turtles, red-eared sliders also demonstrate differences in f_H related to their activity level (Krosniunas and Hicks, 2003). Heart rate increased when red-eared sliders were active, however, in these turtles, the increases in f_H appeared to be constrained to a narrow range set by body temperature (Krosniunas and Hicks, 2003). In the present study, water temperature did not have a significant effect on f_H during different activities, although there was only a minimal temperature difference among the turtles in 2015 (Table 1). Further research is needed to determine if there is a similar effect of activity level on f_H in marine turtles freely diving at sea and if cardiovascular responses to activity at different water temperatures are similarly constrained by narrow f_H ranges.

Heart rate relation to dive duration. Marine mammals and emperor penguins frequently extend aerobic dive durations by reducing f_H , which reduces O_2 consumption in perfusion dependent organs. In emperor penguins and grey seals diving at sea, the slopes of the relationship between heart beats and dive duration begin with a linear relationship similar to that in loggerhead turtles (Fig. 5A); but then the number of heart beats flattens out in dives beyond six and seven minutes,

respectively (Thompson and Fedak, 1993; Wright et al., 2014). This plateau, which is consistent with greater reductions in f_H during longer dives of seals and penguins, was not observed in the loggerhead turtles (Figs. 5A & 5B). The lack of a plateau suggests these turtles did not need to further reduce f_H and O_2 consumption to maintain aerobic metabolism during the long dives in this study. Both this linear increase in total number of heart beats with dive duration and the fact that all dive durations were less than cADLs (Table 1) support the hypothesis that metabolism was primarily aerobic in these turtles.

It is possible that, in loggerhead turtles at sea, f_H decreases further in longer dives. The longest dives in the present study (30-50 mins) were considerably shorter than the longest dives of similarly-sized loggerhead turtles diving at sea (80-150 mins) in comparable temperatures (Hochscheid et al., 2007; Hochscheid et al., 2005). Further, minimum instantaneous f_H during dives in the present study declined slightly, but significantly with dive duration (Fig. 6), raising the possibility that diving f_H may significantly decrease in dives over 50 mins.

However, the shorter dive durations in the present study have several possible explanations with different implications for changes in diving f_H . First, loggerhead turtles in the North Pacific may dive for shorter durations with higher f_H because they have a higher metabolic rate than other populations. North Pacific juvenile loggerhead turtles maintain higher resting metabolic rates than Mediterranean loggerhead turtles, which results in lower cADLs of North Pacific turtles (Hochscheid et al., 2004; Kinoshita et al., 2018) (See Table S1 for differences in \dot{V}_{O_2} and cADLs calculated using equations derived from data of the two populations). Future heart rate studies comparing Mediterranean and North Pacific loggerhead turtles might reveal lower resting heart rates in Mediterranean turtles considering their lower metabolic rate. Second, anaerobic metabolism during dives at sea may contribute to longer dive durations, which would not necessitate lower f_H in longer dives. Marine turtles have a high capacity for anaerobic metabolism, however, anaerobic metabolism is believed to be used during intense activities rather than to extend dive durations (Southwood et al., 2006; Southwood et al., 2003). Third, dives in the present study could be shorter because turtles are diving with a reduced lung volume. To remain at their preferred depth, loggerhead turtles alter their depth to maintain neutral buoyancy or slightly negative buoyancy, which conserves O_2 by reducing the work effort required to move underwater or rest on the ocean floor (Hays et al., 2004; Hochscheid et al.,

2003; Minamikawa et al., 2000). Since turtles could not alter their depth in the shallow tanks, to become slightly negatively buoyant they would have to decrease their inhaled lung volume, reducing the lung O₂ store. As a result, loggerhead turtles diving at sea may have similar f_H , but longer durations as a result of diving with a full lung volume, although extended dive durations were not shown in weighted loggerhead turtles diving in shallow tanks (Hochscheid et al., 2003). Thus, whether dive durations are shorter or longer at sea, f_H may not be lower than in this study because of differences in (1) metabolic rates between populations of loggerhead turtles, (2) preferred metabolic pathways at sea, or (3) diving lung volume. Additional research is needed to investigate f_H in freely diving loggerhead turtles and determine if there is a relationship between at sea dive durations and f_H .

PSI f_H and minimum PSI duration increased after longer dive durations. While loggerhead turtles did not extend dive durations by reducing f_H , they did increase PSI f_H after longer dives (Fig. 7). A faster f_H will reload O₂ stores and unload CO₂ more quickly, minimize time at the surface and allow turtles to maintain high dive-duration-to-surface-interval ratios. While the longest dives did not have the longest PSI durations, there was a clear increase in the minimum PSI duration as dive durations increased (Fig. 2), suggesting additional minimum recovery times were necessary for longer dives. However, the increase was minor as the longest dives only increased the minimum PSI by a few minutes.

Heart rate patterns. The profiles of loggerhead turtle f_H are similar to those of other turtle studies in that underwater resting f_H was lower compared to swimming f_H or eupneic f_H (Table 3). However, it is difficult to draw any other conclusions from comparisons to prior studies because of differences in measurement conditions, turtle size, species and water temperature (Table 3). Despite these differences, resting f_H and swimming f_H in the present study were much lower than in turtles from past studies, whether other turtles were bigger or smaller, in colder or warmer temperatures, or swimming in tanks or at sea. These exceptionally low heart rates were apparently adequate for maintenance of homeostasis out of water or underwater in these captive turtles.

The highest overall f_H in this study occurred when turtles were at the surface. The high PSI f_H was likely driven by ventilation, as well as activity. Activity at the surface included swimming and movements required for breathing. Marine turtles swim with their head underwater, but use their flippers to help lift their head to breathe (Prange, 1976). We could not separate the activity costs from ventilatory costs since turtles did not stop moving during PSIs, preventing a comparison between f_H while floating at the surface and f_H while surface swimming. However, a transient increase in f_H was associated with breathing in green turtles (Butler et al., 1984; Davenport et al., 1982; West et al., 1992). While ventilatory costs may be very low in marine turtles, increased ventilation likely contributes to a higher PSI f_H since PSI f_H increased with longer dives.

Conservation implications. Mortality rates from turtle interactions with fisheries are high, with loggerhead turtles having interactions with the more fisheries than other marine turtle species in the United States (Finkbeiner et al., 2011). Recent discovery of gas emboli in sea turtles suggest turtles may suffer from decompression sickness after being entangled in nets at depth (García-Párraga et al., 2014). While turtles typically lower heart rate during forced submergences (Berkson, 1966), the physiological response to net entanglement is likely different. Our results suggest that, if turtles are struggling in nets, heart rate will increase from vigorous activity, which will result in rapid oxygen depletion. A faster oxygen depletion can contribute to lactate accumulation, and turtles may suffer from lactic acidosis. Further, a higher heart rate will increase pulmonary blood flow which may contribute to increased nitrogen absorption, potentially resulting in the development of gas emboli, recently observed in marine turtles after being entangled in nets (García-Párraga et al., 2018). Future studies designed to increase our understanding of physiological responses to both baseline activities and net entanglement is vital for understanding how to protect and manage marine turtle populations.

Intravascular ECG electrodes resulted in clear ECG records even during activity. ECG signals in 2013 were clear when the turtles were resting on the bottom of the tanks, but became increasingly noisy as the turtle began to move, similar to what was observed in green turtles during movement (Southwood et al., 2003). The noise is likely due to interference from

electromyography signals from muscle contractions during movements as it was highest when turtles were the most active – swimming at the surface. The use of a bipolar pacing catheter in 2015 resolved this problem, providing clear ECG signals during all activities with only rare instances of unreadable signals (Fig S1).

Conclusion

Our results that f_H while resting out of water was no different from f_H during dives or bottom rest indicate that, under the conditions of this study, f_H is not further reduced by submergence. Further, our finding that f_H also remained relatively constant in short and long dives suggests that loggerhead turtles do not rely on further f_H reductions to reduce O_2 consumption and maintain aerobic metabolism during the longer dive durations of this study. Rather, f_H appears driven by underwater activity levels as adjustments in f_H during dives were in line with an exercise response. With their lack of elevated muscle O_2 stores and the apparent absence of reduced muscle blood flow during forced submersions (Lutz and Bentley, 1985), loggerhead turtles must maintain high enough f_H to support routine O_2 consumption, as well as the increased O_2 requirements in muscle during activity. Thus, the increase in f_H during feeding may be at least partially in response to the additional muscle O_2 demands from underwater swimming.

The heart rates we report here highlight the value of recording ECG using self-contained data loggers. Our findings of lower overall heart rates compared to previously measured turtles suggest that in some cases heart rates in other studies may have been affected by the presence of investigators or the turtles being attached to external equipment. The heart rates of undisturbed loggerhead turtles in our study provide a baseline for understanding cardiac responses to resting, surface swimming and underwater feeding. These are key activities for the loggerhead turtle, which is known to rest on the ocean bottom for over seven hours in cold temperatures (Hawkes et al., 2007; Hochscheid et al., 2007), to spend significant time at the surface, (traveling or foraging on surface prey) (Hochscheid et al., 2010; Polovina et al., 2004), and to forage within the water column (Casale et al., 2008; Narazaki et al., 2013). Heart rates at the surface were the highest likely due to increased O_2 demands from continuous swimming and ventilation. $PSI f_H$ increased as dive duration increased. This strategy allows loggerhead turtles to maximize underwater foraging time by minimizing recovery time at the surface.

These results are the first f_H records of undisturbed loggerhead turtles and provide valuable information to assess captive loggerhead turtles as well as to compare to future studies on marine turtles at sea. Whether cardiac responses in loggerhead turtles freely diving at sea are different from the present study awaits further investigation.

List of Symbols and Abbreviations

f_H	heart rate
ECG	electrocardiogram
O ₂	oxygen
Mb	myoglobin
TDR	time-depth recorder
ADL	aerobic dive limit
cADL	calculated aerobic dive limit
\dot{V}_{O_2}	oxygen consumption
T _w	water temperature
A	activity
PSI	post-dive surface interval

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Competing Interests

The authors declare no competing or financial interests.

Author Contributions

Conceptualization: C.L.W., P.J.P.; Methodology: C.L.W., K.S., P.J.P.; Software: C.L.W.; Validation: C.L.W., P.J.P.; Formal Analysis: C.L.W.; Investigation: C.L.W., K.S., P.J.P.; Resources: K.S., P.J.P.; Writing – original draft preparation: C.L.W.; Writing – review and editing: C.L.W., K.S., P.J.P.; Visualization: C.L.W.; Supervision: P.J.P.; Project administration: K.S., P.J.P.; Funding Acquisition: C.L.W., P.J.P.

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Supplementary information

Supplementary information available online at _____

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Figures

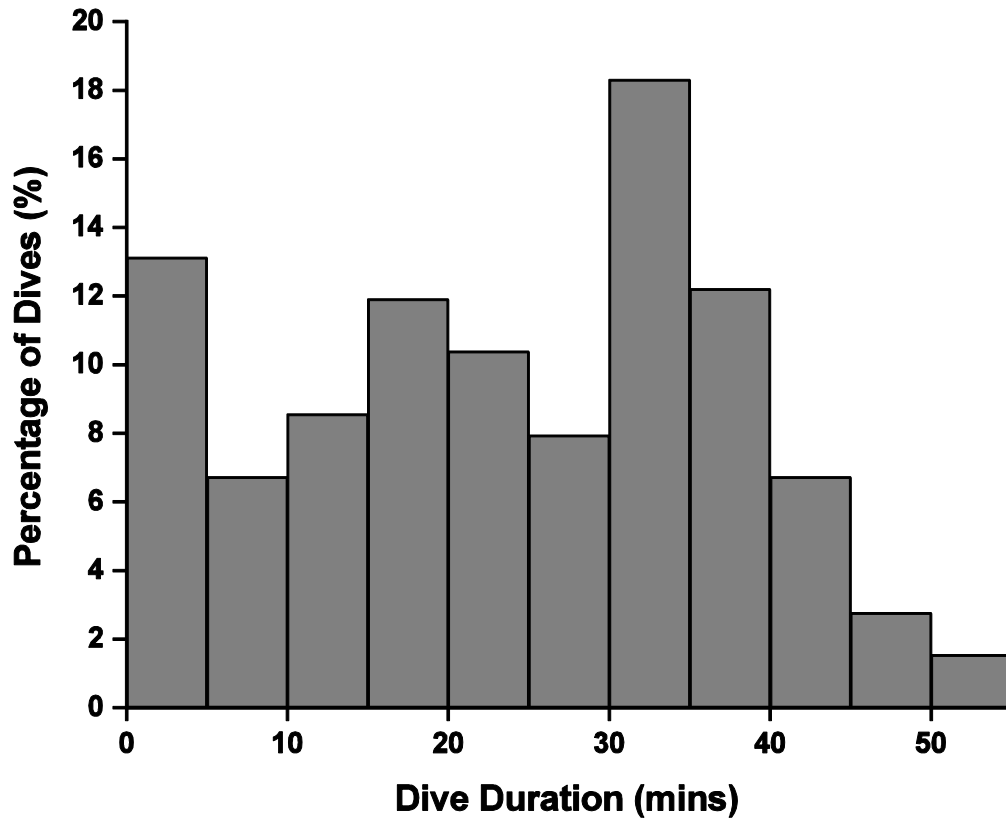


Fig. 1. Frequency distribution of dive durations. (N=8 turtles, 328 dives).

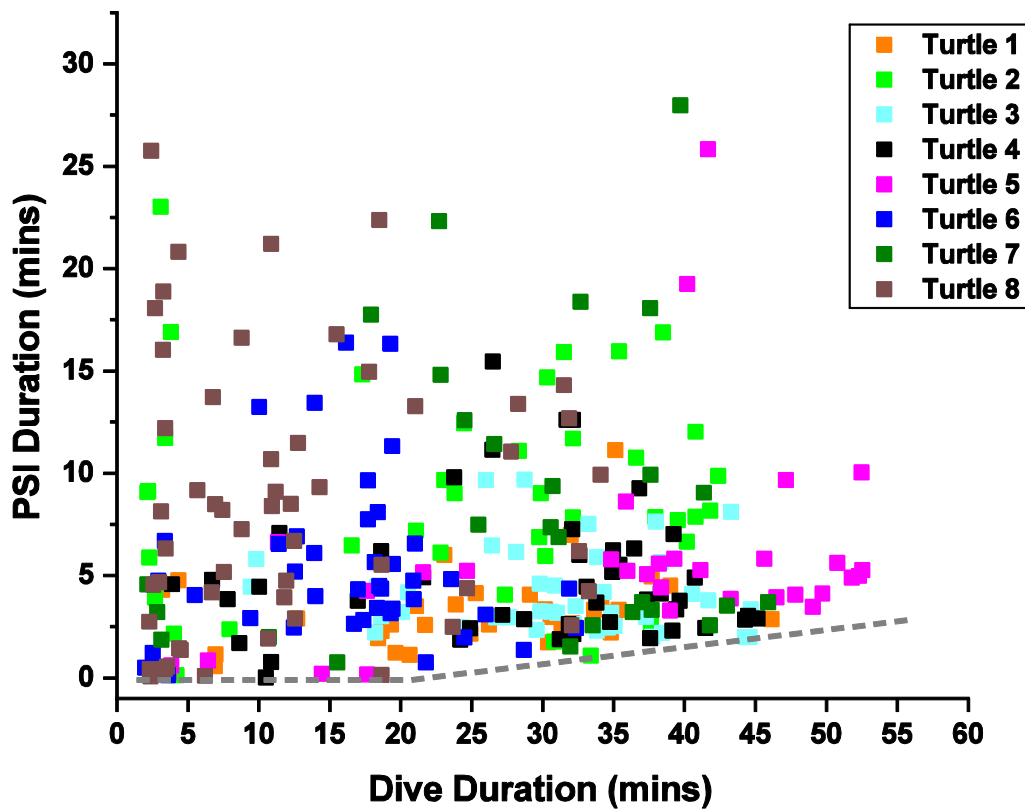


Fig. 2. **PSI duration versus dive duration.** Dives with PSIs in excess of 30 minutes were excluded. Dashed line represents minimum PSI duration, n=8 turtles, 317 dives, PSI = post-dive surface interval.

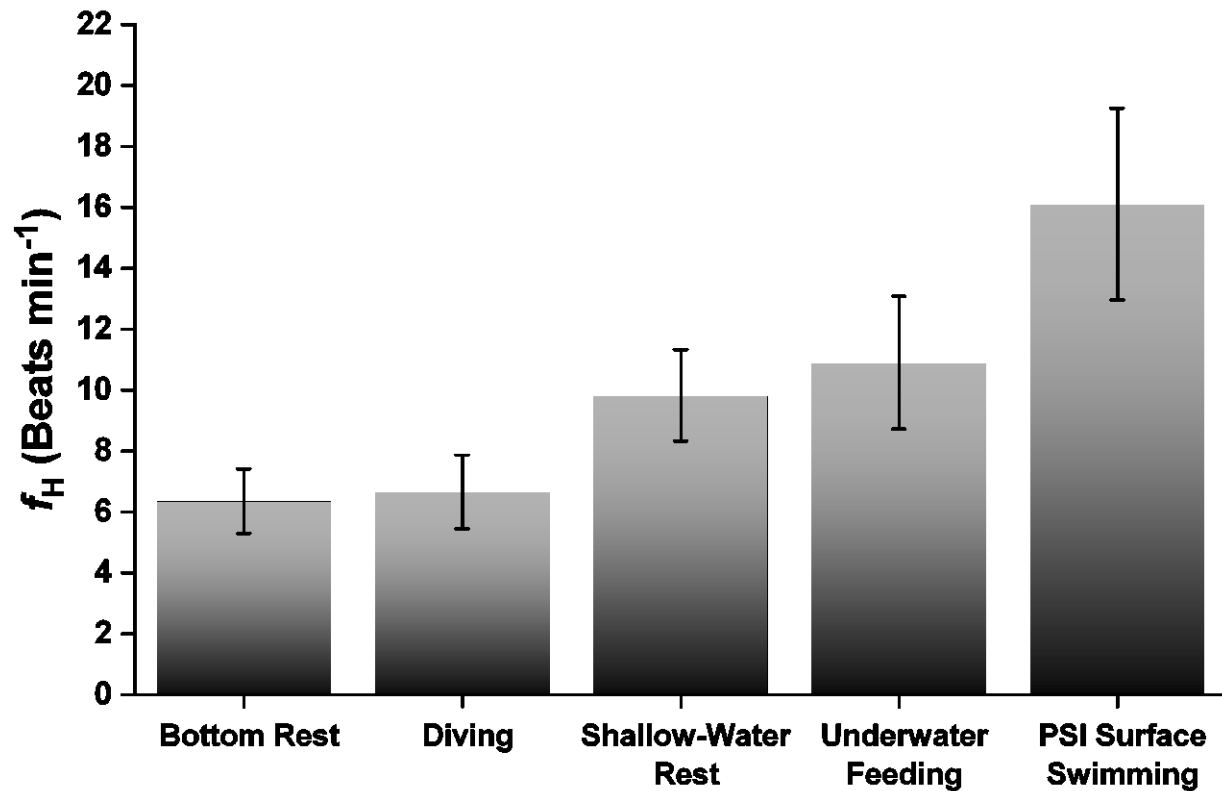


Fig. 3. Mean \pm s.d. heart rate (f_H) for five routine activities of loggerhead turtles from 2015. Bottom rest indicates turtles resting motionless on the bottom of tanks, whereas shallow-water rest occurred when turtles were in shallow water with their head out of water. During underwater feeding, turtles swam (without breathing) after squid placed in the tank. Dives began when the turtle's head was underwater and ended when the turtle raised its head to breathe. Surface swimming includes breathing. N=4 turtles.

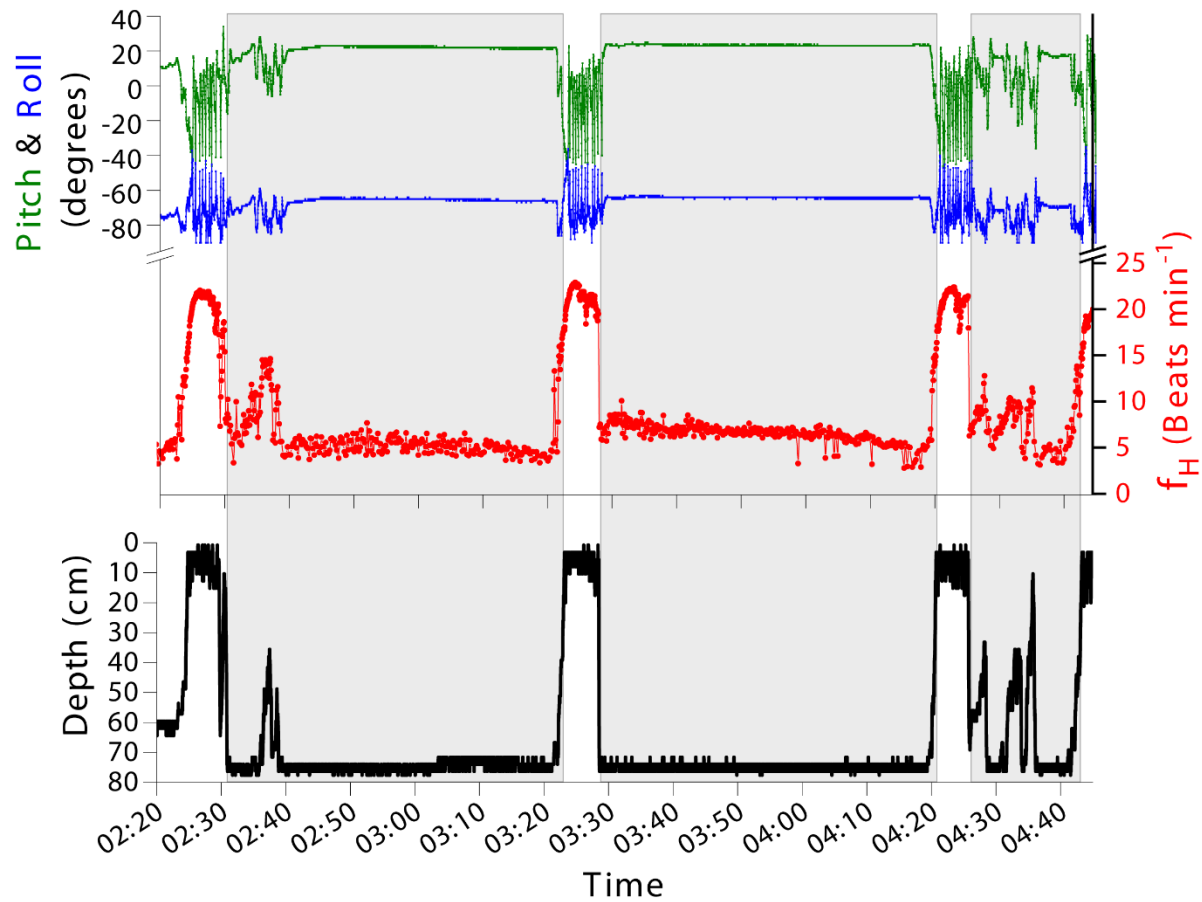


Fig. 4. **Heart rate (f_H , red), pitch (green), roll (blue) and depth (black) profiles during three dives and three surface intervals of Turtle #5 on September 8, 2015.** Shaded areas indicate turtle is submerged. Heart rate during dives is low unless turtle is moving, as indicated by the pitch and roll profiles.

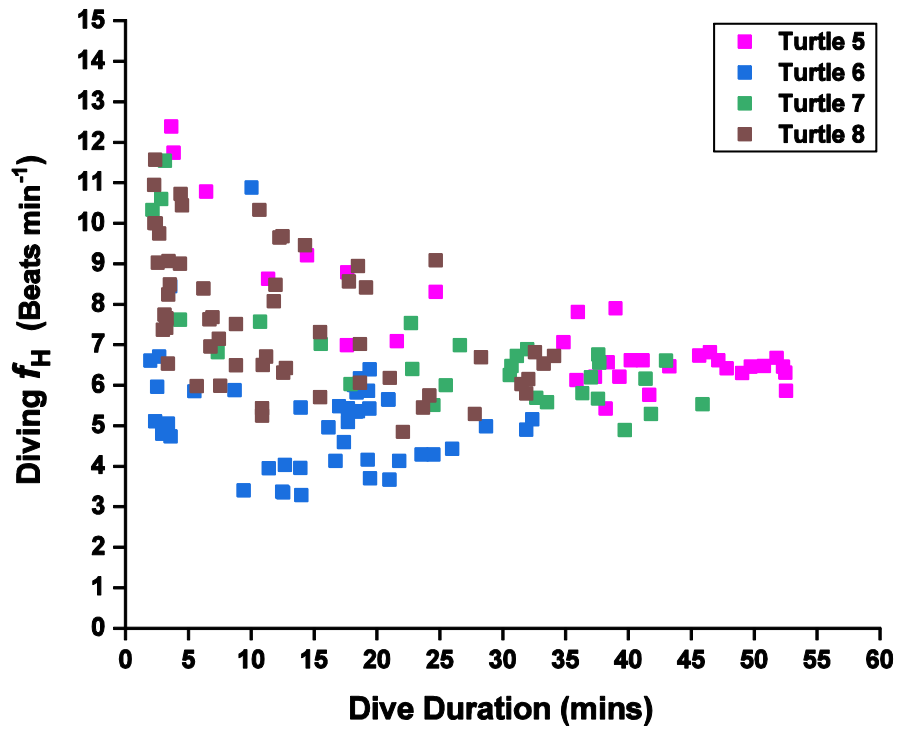
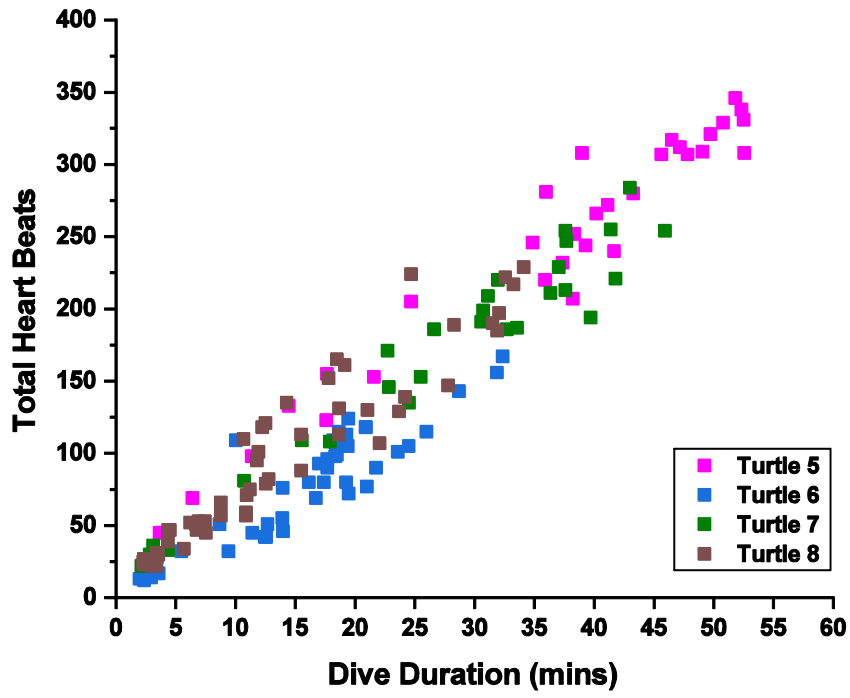


Fig. 5. **Heart rate (f_H) during dives of 2015 loggerhead turtles.** A) Total heart beats versus dive duration. B) Heart rate versus dive duration. Individual turtles are indicated by different colors, n=4 turtles, 164 dives.

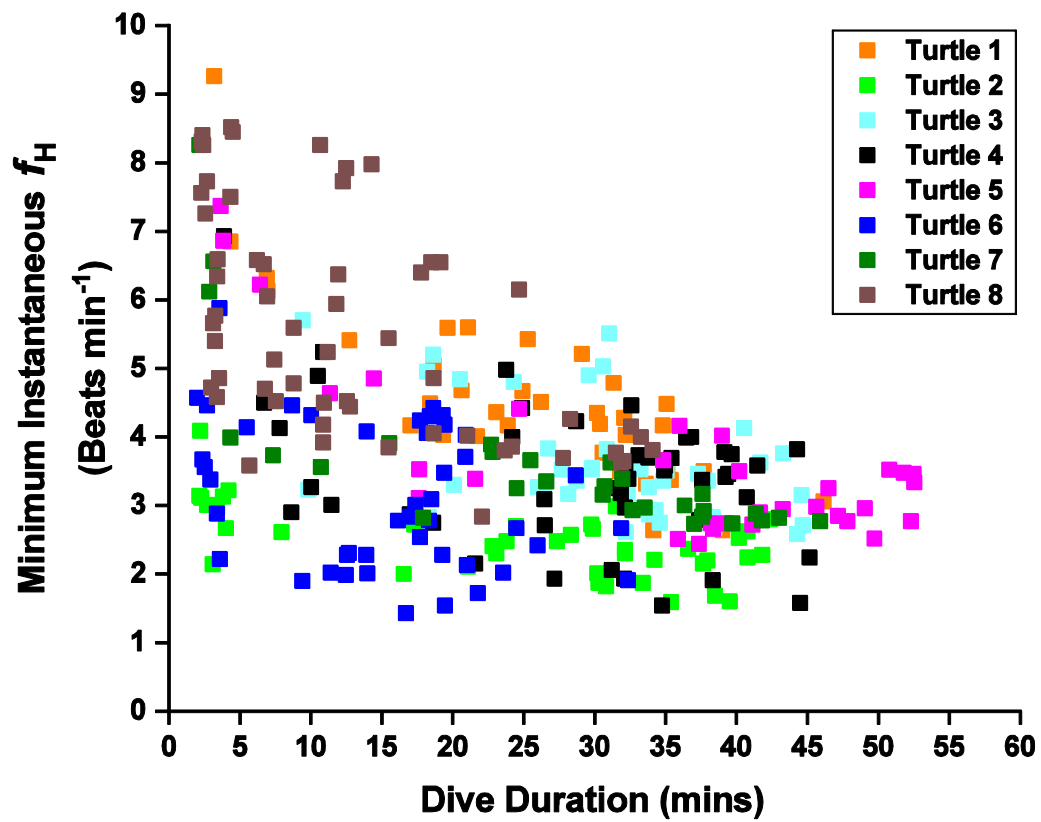


Fig. 6. Minimum instantaneous heart rate (f_H) during dives versus dive duration. Individual turtles are represented by different colors (see legend), $n=8$ turtles, 328 dives.

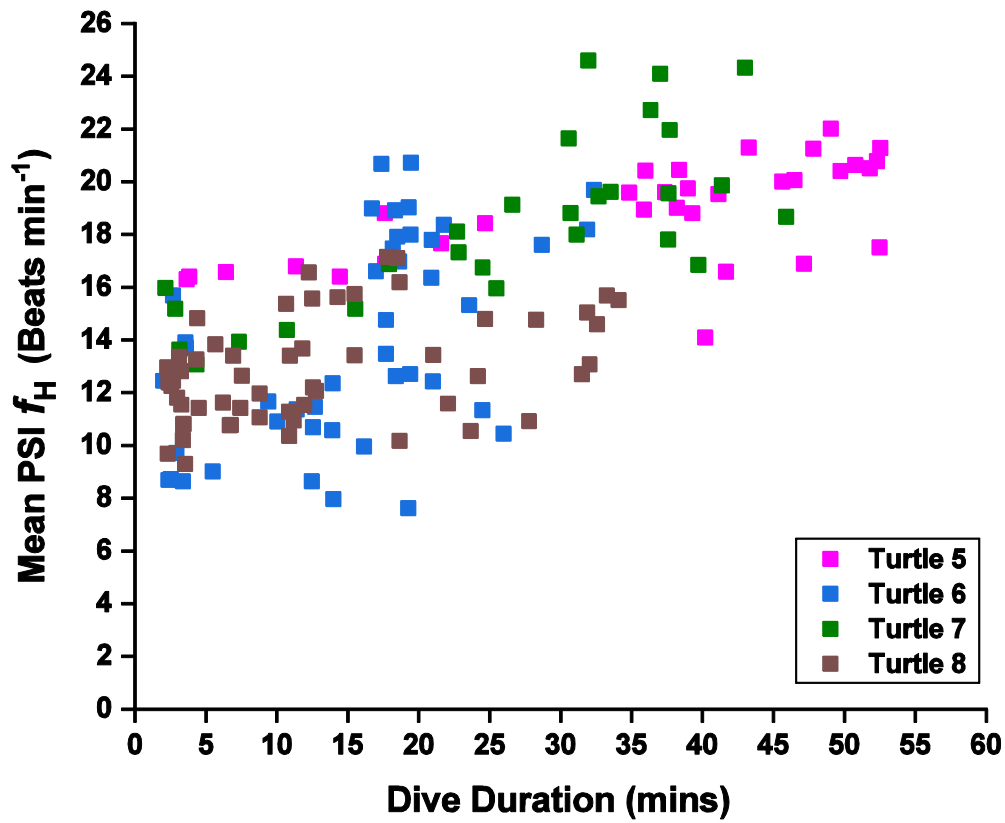


Fig. 7. Mean heart rate (f_H) during surface intervals after dives compared to the duration of the previous dive. Individual turtles are represented by different colors (see legend), $n=4$ turtles, 160 dives, PSI = post-dive surface interval.

Tables

Table 1. Summary of data for each turtle from 2013 (turtles 1-4) and 2015 (turtles 5-8).

Turtle	Mass	Number of Dives & (PSIs)	Mean Dive Duration	Maximum Dive Duration	PSI Duration	cADL Range	Minimum Dive Instant. f_H	Mean Dive Water Temperature
	<i>Kg</i>		<i>Mins</i>	<i>Mins</i>	<i>Mins</i>	<i>Mins</i>	<i>Beats min⁻¹</i>	<i>°C</i>
1	88	36 (35)	25.7 ± 10.2	46.1	3.3 ± 1.9	54.4-115.1	4.5 ± 1.3	22.8 ± 0.8
2	61	41 (39)	24.8 ± 13.7	42.4	8.9 ± 5.0	51.7-109.4	2.5 ± 0.5	22.6 ± 0.3
3	79	41 (41)	31.0 ± 8.7	44.7	4.2 ± 2.1	51.5-109.1	3.6 ± 0.8	23.4 ± 0.8
4	66	46 (45)	29.0 ± 11.3	45.1	4.9 ± 3.3	52.7-111.5	3.4 ± 1.0	22.5 ± 0.8
5	41	32 (32)	35.4 ± 15.3	52.5	5.7 ± 5.1	56.5-119.6	3.6 ± 1.2	19.9 ± 0.2
6	57	45 (44)	15.3 ± 8.0	32.4	5.1 ± 4.0	57.7-122.2	3.1 ± 1.0	20.5 ± 0.5
7	38	29 (27)	26.7 ± 13.5	45.9	8.5 ± 7.2	55.8-118.1	3.6 ± 1.3	19.9 ± 0.7
8	38	58 (57)	13.0 ± 9.8	34.1	8.7 ± 6.5	56.1-118.8	5.6 ± 1.6	19.8 ± 0.9
Grand Mean	58.5 ± 18.9	41.0 ± 9.1	24.1 ± 13.4	42.9 ± 6.7	6.2 ± 5.1	54.5 – 115.4	3.8 ± 1.5	21.4 ± 1.5

Calculated aerobic dive limit (cADL) range for turtles is from 100% to 0% active (Kinoshita et al., 2018). Means are reported ± standard deviation. Mean dive water temperature is water temperature during dives. PSI, post-dive surface interval; f_H , heart rate. N=8 turtles.

Table 2. Heart rate (f_H) data for five conditions and feeding duration for the four 2015 turtles.

Turtle	Diving f_H	Bottom Rest f_H	Shallow-Water Rest f_H	Feeding f_H	Feeding Duration	PSI f_H
	<i>Beats min⁻¹</i>	<i>Beats min⁻¹</i>	<i>Beats min⁻¹</i>	<i>Beats min⁻¹</i>	<i>Mins</i>	<i>Beats min⁻¹</i>
5	7.3 ± 1.7	6.1 ± 0.8	9.5 ± 0.9	8.9 ± 2.5	4.1	18.9 ± 1.9
	<i>(5.4-12.4)</i>	<i>(3.7-7.4)</i>	<i>(7.3-11.7)</i>	<i>(4.9-13.5)</i>		<i>(14.1-22.0)</i>
6	5.2 ± 1.4	5.5 ± 0.6	9.9 ± 2.9	9.8 ± 3.1	5.7	13.9 ± 4.0
	<i>(3.3-10.9)</i>	<i>(4.1-7.7)</i>	<i>(4.5-16.2)</i>	<i>(4.6-14.6)</i>		<i>(7.6-20.7)</i>
7	6.8 ± 1.6	5.8 ± 0.8	11.8 ± 2.6	13.9 ± 3.2	2.5	18.7 ± 3.0
	<i>(4.9-11.6)</i>	<i>(3.9-7.2)</i>	<i>(6.0-16.8)</i>	<i>(5.7-19.7)</i>		<i>(13.6-24.6)</i>
8	7.6 ± 1.7	7.8 ± 3.4	8.2 ± 0.7	11.0 ± 1.5	1.9	12.9 ± 2.0
	<i>(4.9-11.5)</i>	<i>(5.0-15.1)</i>	<i>(6.6-10.2)</i>	<i>(7.9-13.2)</i>		<i>(9.3-17.2)</i>
Grand Mean	6.7 ± 1.1	6.4 ± 1.0	9.9 ± 1.5	10.9 ± 2.2	3.6 ± 1.7	16.1 ± 3.2

There was one bottom rest, shallow-water rest and feeding event per turtle. The numbers of dives and PSIs are different for each turtle and specified in Table 1. For f_H columns, the first line is the mean f_H ± standard deviation and the second line is the range of f_H italicized and in parentheses. PSI, post-dive surface interval; f_H , heart rate. N=4 turtles.

Table 3. Heart rate (f_H) of different marine turtle species during underwater rest and swimming or breathing.

Marine Turtle Species	Mass (kg)	Water Temp. (°C)	Study Conditions	Sample Size	Underwater Rest f_H (Beats min⁻¹)	Swimming/ Eupneic f_H (Beats min⁻¹)	Reference
<i>Loggerhead</i>	38-88	20-23	<i>Data logger in tanks</i>	8	6.4	10.9	<i>Present study</i>
Loggerhead	11.8	19.3-22.5	Flipper blood flow study	1	18	19	Hochscheid et al., 2002
Loggerhead	25	15.5	Attached to cord in tank	1	7.5	15	Lanteri et al., 1980
Leatherback	172-377	25-27.5	Freely diving at sea	5	NA	17.4	Southwood et al., 1999; Southwood et al., 2005
Green (Winter)	19-43	17	Data logger in tanks	3-5	10.2	12.9	Southwood et al., 2003
Green (Summer)	19-43	26	Data logger in tanks	3-5	19.6	24.0	Southwood et al., 2003

Heart rate from present study is lower than marine turtles in other studies. Note differences in turtle size, water temperature, and study conditions.
 f_H , heart rate

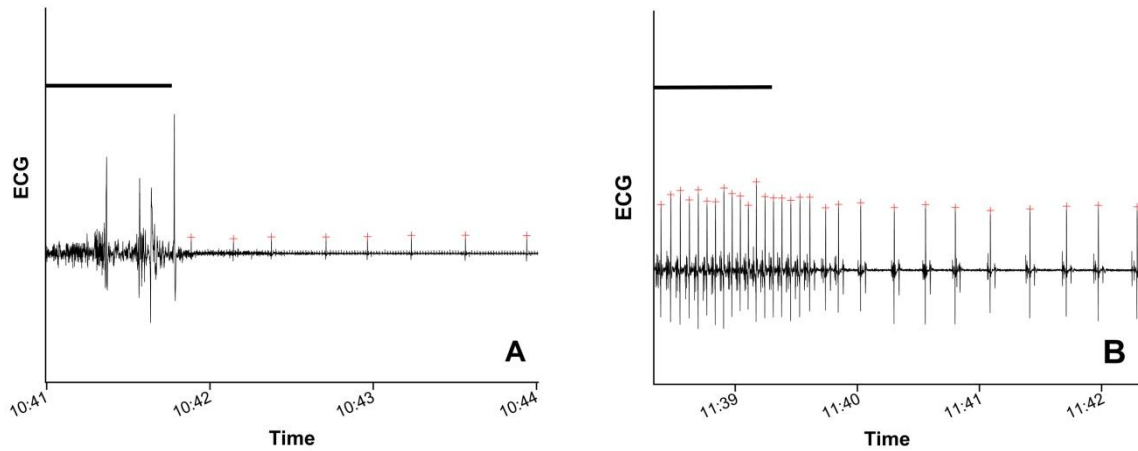


Figure S1. Example of ECG signals from A) Turtle 2 on Sept. 18, 2013 and B) Turtle 6 on Sept. 12, 2015. R waves are denoted by a red plus sign above the wave and the black bars above the ECG signals indicate when the turtle was at the surface. ECG signals were very difficult to read in 2013 (A) during surface periods, but legible during dives (~10:42 – 10:44). However, R waves were quite legible in 2015 (B), even during surface periods.

Table S1. Oxygen (O₂) stores, \dot{V}_{O_2} and calculated aerobic dive limits (cADLs) for eight turtles using different equations.

Turtle	Mass	O ₂ Stores	Water Temp.	\dot{V}_{O_2}		cADL		\dot{V}_{O_2}		cADL	
				100% Active Kinoshita	0% Active Kinoshita	100% Active Kinoshita	0% Active Kinoshita	Hochscheid	Hochscheid		
	Kg	mL O ₂	°C	mL O ₂ min ⁻¹	Mins	mL O ₂ min ⁻¹	Mins	mL O ₂ min ⁻¹	Mins	mL O ₂ min ⁻¹	Mins
T1	88	1953.6	22.8	35.94	54.4	16.98	115.1	12.691	153.9		
T2	61	1354.2	22.6	26.21	51.7	12.38	109.4	10.783	125.6		
T3	79	1742.7	23.4	34.02	51.5	16.07	109.1	13.482	129.3		
T4	66	1454.1	22.5	27.81	52.7	13.14	111.5	10.873	133.7		
T5	41	910.2	19.9	16.11	56.5	7.61	119.6	5.954	152.9		
T6	57	1265.4	20.5	21.93	57.7	10.36	122.2	7.398	171.0		
T7	38	843.6	19.9	15.12	55.8	7.14	118.1	5.797	145.5		
T8	38	843.6	19.8	15.04	56.1	7.10	118.8	5.700	148.0		

Two calculations are based on the \dot{V}_{O_2} equation from Kinoshita et al.: $\dot{V}_{O_2} = 0.1098 \exp(0.0581T_w + 0.0075A)$, where T_w is the water temperature in Celsius, A is the percentage of time turtle was active and units are expressed as ml O₂ min⁻¹ kg^{-0.83}. The first \dot{V}_{O_2} and cADL are assuming the turtle are active 100% of the time and the second set assumes the turtles are active 0% of the time (Kinoshita et al., 2018). The second equation is from Hochscheid et al.: $\ln \dot{V}_{O_2} = -2.87 + 0.168 T_w + 0.353 \ln M_b$, where M_b is turtle mass (Hochscheid et al., 2004). The Kinoshita equation is based on data collected from North Pacific loggerhead turtles and the Hochscheid equation is based on data collected from Mediterranean loggerhead turtles (Hochscheid et al., 2004; Kinoshita et al., 2018).