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Physiology

# All puffed out: do pufferfish hold their breath while inflated?

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The inflation response of pufferfishes is one of the most iconic predator defence strategies in nature. Current dogma suggests that pufferfish inflation represents a breath-holding response, whereby gill oxygen uptake ceases for the duration of inflation and cutaneous respiration increases to compensate. Here, we show that the black-saddled pufferfish (*Canthigaster valentini*) has an excellent capacity for oxygen uptake while inflated, with uptake rates increasing to five-times that of resting levels. Moreover, we show that this species has negligible capacity for cutaneous respiration, concluding that the gills are the primary site of oxygen uptake while inflated. Despite this, post-deflation recovery of aerobic metabolism took an average of 5.6 h, suggesting a contribution of anaerobic metabolism during pre-inflation activity and during the act of ingesting water to achieve inflation.

## 1. Introduction

When considering the vast suite of adaptations that exist in the animal kingdom to avoid predation, it is difficult to overlook the impressive repertoire of the pufferfishes (family Tetraodontidae). While pufferfish flesh contains one of the most potent toxins known to science [1], the common name for this group of fishes stems from their remarkable and iconic ability to ‘puff’ themselves into a large ball when threatened. The inflation response is made possible through rapid gulping of water into a distensible stomach [2–5], which stretches the exceptionally elastic skin and promotes the erection of small spinules embedded in the dermis [6]. The final result is a spiny ball that can be three- to four-times the resting volume of the fish and not easily ingested [2,7,8].

As a non-evasive form of defence, the success of the puffing response relies upon the capacity of the pufferfish to remain inflated for longer than the attention span of the would-be predator. Interestingly, reports in the scientific literature suggest that pufferfish inflation may be a breath-holding response, whereby oxygen uptake and waste excretion across the gills are interrupted for the duration of the inflation event (which can last tens of minutes). Indeed, the opercula openings of the obscure pufferfish (*Takifugu obscurus*) have been reported to remain closed throughout the inflation period [8], while the long-spined porcupinefish (*Diodon holocanthus*, a close relative of the pufferfishes and a member of the tetraodontid sister family Diodontidae) has been described as ‘appearing to hold its breath’ once inflated [7]. If these reports are accurate, it can be speculated that an oxygen limitation (i.e. hypoxaemia) may ultimately set the maximum duration of the inflation response.

With this in mind, it has been proposed that cutaneous respiration may play a compensatory role for pufferfishes during inflation [6]. A study of the green pufferfish (*Tetraodon fluviatilis*) [6] noted that the spinules erect through invaginations in the epidermis and remain covered by a thin layer of loose connective tissue rich in blood capillaries, subsequently concluding that this creates a thin gas diffusion pathway which may allow for significant cutaneous respiration while inflated.

In light of the speculation and mystery that surrounds pufferfish inflation, this study used the black-saddled pufferfish (*Canthigaster valentini*) and novel approaches to determine (i) whether oxygen transport across the gills is maintained during the inflation response, (ii) the metabolic costs of the inflation response and the time required to recover, and (iii) the role of cutaneous respiration in satisfying oxygen requirements during the inflation response.

## 2. Material and methods

### (a) Fish collection and holding conditions

Black-saddled pufferfish (*C. valentini*) were collected on SCUBA from the Lizard Island and Cairns region of the Great Barrier Reef, Australia. Notably, most fish underwent 5–10 s of burst swimming and then an inflation response as they were captured in hand nets. After transport to an indoor aquarium facility at the Australian Institute of Marine Science, fish were randomly housed two fish per 20 l tank with the fish separated by a solid divider down the middle. The tanks were supplied with particle-filtered and UV-sterilized seawater from nearby Cleveland Bay and were maintained at a temperature of 25°C for four weeks prior to experimentation. The fish were kept on a 13 L : 11 D cycle and fed to satiation daily on thawed prawn meat and Hikari algae wafers.

### (b) Whole-animal respiration

#### (i) Respirometer set-up

Intermittent flow-through respirometry was used for all oxygen consumption rate ($\dot{M}_{O_2}$) measurements following best practices outlined in [9]. Individual *C. valentini* were placed into custom-built rectangular respirometers (0.63 or 1.47 l, depending on fish size) which were submerged in a water bath maintained at 25°C. Each respirometer was equipped with a closed-circuit recirculation loop (powered by an inline pump) containing a sensor that measured respirometer oxygen levels continuously at 0.5 Hz (FireSting $O_2$; PyroScience, Germany). The respirometers were automatically flushed with aerated water for 5 min in every 10 min period to maintain oxygen levels above 80% air saturation, and $\dot{M}_{O_2}$ was measured from the rate of decline in water oxygen saturation between flush cycles. The fish were allowed to recover from handling and to acclimate to the respirometers overnight (minimum of 12 h). Background $\dot{M}_{O_2}$ was measured in empty respirometers before and after each trial, and a linear change was assumed when correcting fish $\dot{M}_{O_2}$ (correction always less than 10% of fish $\dot{M}_{O_2}$).

#### (ii) Inflation $\dot{M}_{O_2}$ and post-deflation recovery

Following overnight acclimation, experiments were conducted to quantify the $\dot{M}_{O_2}$ of fish during inflation (six males, two females, mean mass 19.27 ± 3.93 g, fasted for 48 h). To induce inflation, each respirometer was opened individually and a fitting comprising a soft piece of vinyl tubing was placed on the inlet of the closed-circuit recirculation loop to act as a suction piece. The suction piece included two holes either side of the tubing to allow a sufficient flow of water through to the oxygen sensor while still providing light suction at the end of the piece to simulate the mouth of a predator. Each fish was manually placed on the suction piece dorso-laterally and squeezed gently to stimulate inflation. Once inflated (typically after 5–10 s), the respirometers were sealed immediately and the flush pump switched off to commence $\dot{M}_{O_2}$ measurements. The duration of inflation was monitored by keeping a close eye on the fish through the semi-transparent lids of the respirometers, and the flush pump was switched on intermittently when required to ensure respirometer oxygen levels remained above 80% air saturation. Following deflation, the flush pump was placed back on the automated system and $\dot{M}_{O_2}$ was recorded for 24 h to monitor recovery. Each fish was considered to have reached full recovery once $\dot{M}_{O_2}$ had stabilized for three consecutive measurements at pre-inflation values (i.e. within 2 s.d. of the mean of the lowest six $\dot{M}_{O_2}$ values measured in the 3 h period immediately prior to the commencement of inflation experiments).

#### (iii) Resting $\dot{M}_{O_2}$

Resting $\dot{M}_{O_2}$ was calculated for each fish as the mean of the lowest 10% of $\dot{M}_{O_2}$ values (from more than 200 measurements) obtained throughout the entire approximately 36 h respirometry protocol after excluding (i) measurements taken within 2 h of respirometer entry, (ii) measurements during inflation and within 2 h following deflation, and finally (iii) outliers, which were considered to be greater than 2 s.d. from the mean of these values [10].

### (c) Cutaneous respiration

Fish were given 13 weeks to recover from the initial $\dot{M}_{O_2}$ trials before measurements of cutaneous respiration were obtained using similar techniques to those outlined above. Using a subset of four fish (two males, two females, mean mass 13.57 ± 3.38 g), each individual was attached to a suction piece within an open chamber and stimulated to inflate. A small custom-built respirometer was used to measure cutaneous oxygen uptake ($\dot{M}_{O_2\ \mathrm{cutaneous}}$). The respirometer (35 or 45 ml, depending on fish size) was constructed using a small plastic vial equipped with an inlet and outlet fitting attached to a pump-driven recirculation loop containing an oxygen sensor. The open-end of the vial (1.77 or 5.73 $cm^2$) was pressed against the ventro-lateral skin of the fish (equivalent to more than 10% of total fish surface area) throughout the entire inflation event, and $\dot{M}_{O_2\ \mathrm{cutaneous}}$ was calculated from the rate of decline in water oxygen saturation. A slightly negative pressure within the respirometer ensured a water-tight seal against the (elastic) skin.

## 3. Results

All individual *C. valentini* remained quiescent in respirometers when left undisturbed and the mean (±s.e. herein) resting $\dot{M}_{O_2}$ was 1.33 ± 0.13 $mgO_2\ min^{-1}\ kg^{-1}$. Inflation was successfully stimulated in all individuals following a 5–10 s period of activity within the respirometer as the fish attempted to avoid handling. The inflation event was characterized by approximately a fourfold increase in $\dot{M}_{O_2}$ above pre-inflation levels (to 6.64 ± 0.88 $mgO_2\ min^{-1}\ kg^{-1}$; i.e. approximately a fivefold increase above resting levels), which declined progressively throughout the inflation period of 3.7–18.0 min (mean 10.1 ± 1.6 min; figure 1). All individuals had clearly visible opercula movements during the inflation period.

Upon deflation, there was no discernible dip in respirometer oxygen levels to suggest that oxygen had been extracted across the stomach from ingested water. $\dot{M}_{O_2}$ increased sharply as the fish broke free of the suction piece before slowly recovering to resting levels over a period of 5.6 ± 1.2 h (figure 1). There was no relationship between inflation duration and the recovery of $\dot{M}_{O_2}$ to pre-inflation levels (measured as excess post-deflation oxygen consumption; see the electronic supplementary material), suggesting that there was no oxygen debt acquired while the fish were inflated. Instead, the oxygen debt may have accumulated during pre-inflation activity and water ingestion. $\dot{M}_{O_2\ \mathrm{cutaneous}}$ was measured continually during 2.4–18.9 min

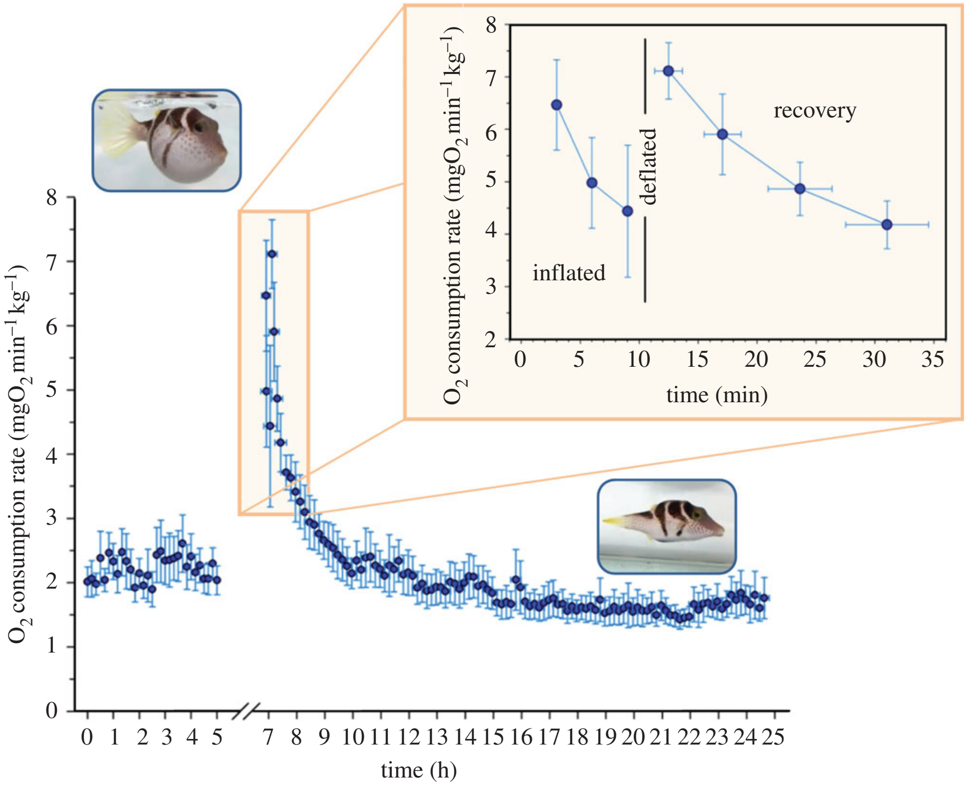


**Figure 1.** Oxygen consumption rates ($\dot{M}_{O_2}$) of black-saddled pufferfish (*C. valentini*) prior to inflation, during inflation and throughout the post-deflation recovery period. The orange box highlights the period of inflation activity, with the inset showing a higher resolution trace of the mean $\dot{M}_{O_2}$ during the inflation period. $N = 8$ for all points except at 6 min during inflation ($N = 6$) and at 9 min during inflation ($N = 4$). Values are means ± s.e.

bouts of inflation and was essentially undetectable, averaging only $1.21 \times 10^{-4} \pm 3.03 \times 10^{-5}$ $mgO_2\,min^{-1}\,cm^{-2}$ (i.e. approx. 0.001% of fish $\dot{M}_{O_2}$ during inflation).

## 4. Discussion

The black-saddled pufferfish, *C. valentini*, demonstrated an excellent capacity for oxygen uptake while inflated, achieving uptake rates well above resting levels. Moreover, the oxygen uptake occurred almost exclusively across the gills rather than via cutaneous pathways. These results show for the first time that the inflation response of pufferfishes is not an anaerobic, breath-holding response as previously suggested [7,8].

The inflation response is thought to be made possible by strong oesophageal and pyloric sphincters. These sphincters are located at the opening between the oesophagus and the stomach, and the opening between the stomach and the intestine, and have been highlighted in early anatomical documentation as the key to keeping ingested water in the pufferfish stomach [11,12]. Indeed, the northern pufferfish (*Sphoeroides maculatus*) was able to maintain a distended state even when the opercula valves were held open or removed [11]. Combined with data from this study, these historical observations suggest that the sphincters in the digestive tract of pufferfishes enable the buccal cavity to remain isolated and the gills to remain functional during inflation.

The very elevated level of $\dot{M}_{O_2}$ achieved by *C. valentini* during inflation and the independence of recovery time from inflation duration (see the electronic supplementary material) provide evidence that the act of remaining inflated does not acquire an oxygen debt. Nevertheless, the post-deflation metabolic recovery period was substantial (mean 5.6 h), suggesting that the activities undertaken prior to the inflation event are at least partly fuelled by anaerobic pathways (figure 1). It is likely that the post-deflation oxygen debt is largely attributed to the 5–10 s of pre-inflation activity associated with preliminary escape attempts, combined with the act of gulping water into the stomach to achieve inflation. In support of this idea, the long-spined porcupinefish was fatigued and could no longer inflate after three to eight successive inflation events [2]. Additionally, the same species was found to require more time for inflation and deflation with every additional event [7]. Clearly, a compromised ability to inflate following successive inflation events would be detrimental to predator evasion and thus impact the fitness and survival of pufferfishes in the natural environment. The brief period of pre-inflation activity documented in this study is ecologically relevant, as similar escape behaviours were witnessed in wild fish prior to inflation during specimen collection.

In sum, we have proven that pufferfishes have the capacity for high rates of oxygen transport across the gills while performing the iconic inflation response. Nonetheless, the inflation event as a whole is energetically taxing, elicits a substantial oxygen debt, and may compromise aerobic activities and subsequent predator avoidance during the post-deflation recovery period. It is possible that the gradual fatigue of the muscles controlling the oesophageal and pyloric sphincters could limit the duration of inflation, a hypothesis that could be addressed by examining excitation–contraction coupling of *in situ* muscle preparations at different stimulation frequencies.

Ethics statement. All experiments were conducted in compliance with the James Cook University Animal Ethics Committee (A1896).

Data accessibility. Data are available in electronic supplementary material and upon request from the authors.

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