The ventilatory, cardiac and behavioural responses of resting cuttlefish (*Sepia officinalis* L.) to sudden visual stimuli

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Summary

When startled, some animals reduce ventilation rate and heart rate, and become motionless. The function of this response, if any, remains unknown. We used non-invasive ultrasound imaging to monitor the ventilatory, cardiac and postural responses of cuttlefish exposed to sudden visual stimuli. Simultaneously, we recorded cuttlefish behaviour using an overhead video camera. Upon presentation of the sudden visual stimulus (rapidly approaching bird cut-out), cuttlefish rapidly changed the colour and the texture of their skin, taking on characteristics of the Deimatic Display. Cuttlefish also became motionless (behavioural freezing), hyperinflated their mantles, and decreased their ventilation rate and heart rate. We found no evidence of a relationship between the intensity of the Deimatic Display and the intensity of any other measured parameter. Ventilation rate decreased during behavioural freezing. Hyperinflation of the mantle was most intense in preparation for and during behavioural freezing. Heart rate decreases occurred during mantle hyperinflation and were greatest in animals showing the most hyperinflation. Decreased heart rate may not be adaptive *per se*. Instead, it might be a product of the unusual arrangement of the cuttlefish peripheral vasculature, which could be compressed during mantle hyperinflation. By filling the mantle with water (hyperinflation), this response to sudden stimuli may help cuttlefish prepare for possible flight by jet propulsion, which often follows the Deimatic Display.

Introduction

It is well-accepted that animals increase ventilation rate and cardiac output after a sudden stimulus (Wingfield, 2003). However, animals across many taxonomic groups have an alternate response to sudden stimuli. The alternate response includes decreased ventilation rate, decreased cardiac output and behavioural freezing (Table 1). Several animals engage in either the traditional fight-or-flight response or the alternate response, depending on the context. For example, if an alligator (Smith et al., 1974) or a burrowing mammal (Smith and Woodruff, 1980; Smith et al., 1981) is cornered in the open, it shows the traditional fight-or-flight response; its heart rate increases, and it fights. However, when the same animal is allowed to hide underwater (alligator) or in its den (burrowing mammal) when frightened, it shows an alternate response; its heart rate decreases and it becomes still.

In response to rapidly approaching objects, some cephalopods, e.g. *Octopus vulgaris* (Wells, 1980; Wells et al.,

1987) and the cuttlefish *Sepia officinalis* (Chichery, 1980), show an alternate response. In cuttlefish, the response includes a transient decrease in ventilation rate and adoption of the Deimatic Display (Table 1). The cephalopod Deimatic Display includes behavioural freezing, rapid changes in skin colour and texture, and body flattening (Hanlon and Messenger, 1996) (e.g. Fig. 1). It is part of a larger category of behaviour called deimatic behaviour. Deimatic behaviour is thought to act as '*threat, startle, frightening or bluff behaviour [that] in most cases serves to make a predator hesitate during the close approach phase of attack*' (Hanlon and Messenger, 1996, p. 79). If deimatic behaviour does not stop the approach of the object or predator, the cephalopod typically jets away by powerfully forcing water from its mantle (Hanlon and Messenger, 1996).

Unlike the function of the cephalopod Deimatic Display, the function of cardiac and ventilatory decreases after sudden stimuli is unknown. It is surprising that ventilation rate and

Table 1. *Examples of the alternate reaction in selected animals across taxonomic groups*

Species	Behaviour	Heart rate	Ventilation	Reference
White-tailed deer fawn (*Odocoileus virtinianus*)	'Freezing'	Decreased by 60±1.4% for 23±1.8 s	Stopped in 72% of trials	Jacobsen, 1979
White Carneaux pigeon (*Columba livia*)	Not reported	Decreased by 6% for 4 s	Not reported	Cohen and MacDonald, 1971
American alligator (*Alligator mississippiensis*)	'Freezing'	Decreased by at least 84% for up to 15 min	Stopped	Smith et al., 1974
Anuran amphibian (*Rana pipiens*)	'Freezing'	Heart missed a beat	Not reported	Laming and Austin, 1981
Tilapia (*Oreochromis mossambicus*)	'Freezing'	Arrhythmia for 10–75 s with arrest for 13.8–18.5 s	Stopped	Barham et al., 1985
Hermit crab (*Dardanus arrosor*)	'Alert pattern'	Cardiac arrest for 1–10 s	Paused scaphognathite irrigation	Cuadras, 1980
Common octopus (*Octopus vulgaris*)	Deimatic Display	Arrhythmia for approximately 30 s	Not reported	Wells et al., 1987
Common European cuttlefish (*Sepia officinalis*)	Deimatic Display	No change	Stopped or slowed for 1–6 s	Chichery, 1980

heart rate fall before possible flight. *Octopus* escape jetting is likely anaerobic (O'Dor and Webber, 1991) and limited by the accumulation of oxygen debt (Wells et al., 1987). Because resting coleoid cephalopods remove almost all the oxygen from their blood during its circuit through the body (Withers, 1992), reduced blood flow or reduced oxygen exchange will force them into anaerobic metabolism. It seems maladaptive to start accumulating oxygen debt, and thereby limit the duration of escape jetting, before escape jetting has started.

In other animals, the adaptive function of bradycardia and decreased ventilation after sudden stimuli is also unknown. Nevertheless, the prevalence of an alternate reaction across vertebrate and invertebrate groups (Table 1) invites hypotheses that assume it has a universally adaptive function. Four major hypotheses explaining the decreases in cardiac and ventilatory function during the alternate response have arisen from the vertebrate literature. They are summarized in Table 2. Unfortunately, because many vertebrate studies simultaneously monitor only two or three aspects of the alternate response (Cuadras, 1981), there is no conclusive evidence about its function in a given vertebrate, let alone if it is similar to that of other animals.

We can monitor ventilation and heart rate in cuttlefish (*S. officinalis*) non-invasively using ultrasound (King et al., 2005). Simultaneously, we can monitor behaviour using an overhead video camera. The goal of this study was to simultaneously quantify the behavioural, postural, cardiac and ventilatory responses of cuttlefish to sudden stimuli. This information will help us to determine the function of the alternate response in cephalopods and possibly suggest a plausible function for it in other animals.

Materials and methods

General housing conditions and experimental set-up

Cuttlefish were housed as described previously (King et al., 2005). Briefly, we obtained six juvenile, cultured *Sepia officinalis* L. from the National Resource Center for Cephalopods, Galveston, Texas, USA. In the Dalhousie Aquatron, we housed two visually isolated cuttlefish in each home tank. Experiments were performed on sexually mature male and female cuttlefish, 15.5–18.5 cm in mantle length, between February and March 2003. Water temperature was 15°C both in the home and in the experimental tanks.

Using the experimental tank described previously (King et al., 2005), we monitored ventilation rate and heart rate using an ultrasound machine and a 5 MHz convex array ultrasound transducer (Ultramark 4 plus, Advanced Laboratory Technologies, Bothell, Washington, USA). Ultrasound videos (sonograms) were recorded on Hi-8 videotape. One cuttlefish
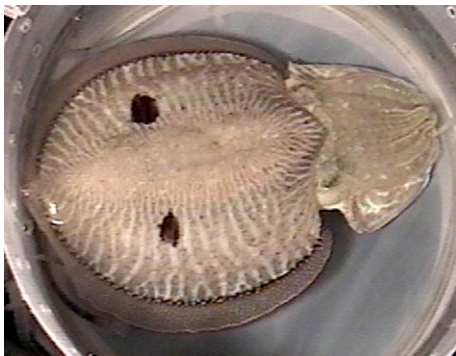

Fig. 1. An example of the Deimatic Display seen in our experiments. This animal showed behavioural freezing, paired Mantle spots (that look like eyes), dark elements on the fins, Smooth mantle, Mantle widening and Arm spreading. It did not show pronounced Mantle paling. Fig. 2A shows the same cuttlefish at rest.

Table 2. *Hypotheses from the vertebrate literature explaining lowered ventilation rate and heart rate after sudden stimuli*

Hypothesis	Reference
(1) Decreased heart rate, decreased ventilation rate and behavioural freezing reduce movement and noise from the animal. This helps it hide from predators.	Jacobsen, 1979; Barham et al., 1985
(2) Ventilation rate and heart rate slow because metabolic rate drops. Lowered metabolic rates allow diving and burrowing air-breathing vertebrates to remain hidden underwater or in a hypoxic den for longer.	Smith and Woodruff, 1980; Smith et al., 1981
(3) In preparation for flight, blood is redistributed from vegetative to locomotory muscles. This causes a reflexive drop in heart rate.	Laming and Savage, 1980; Laming and Austin, 1981
(4) Heart rate drops to reduce blood pressure. This protects delicate tissues and capillaries during the subsequent increase in blood pressure at the onset of locomotion.	Ide and Hoffmann, 2002; Cooke et al., 2003

was present in the experimental tank at a time. To reduce disruption to the cuttlefish during experiments, the experimental tank was divided into an inner and outer compartment. The water-filled space between the compartments allowed the transducer to be operated without disturbing the cuttlefish. To avoid acoustic blocking from the cuttlebone, we insonated cuttlefish from below, through the acoustically transparent plastic bottoms of both the inner and outer compartments. In separate experiments, we noted that water remained well aerated in the inner tank when a cuttlefish was present (>90% O_2 saturation).

To visually isolate the cuttlefish from the rest of the room during experiments, an opaque plastic curtain surrounded the experimental tank. A camcorder (CCD-TR910 NTSC, Sony, Tokyo, Japan) above the tank and connected to a remote monitor (Trinitron, Sony), enabled us to monitor cuttlefish behaviour and to record it on Hi-8 videotape during experiments. Sonograms and behavioural videos were synchronized using a pre-determined audio cue recorded on both tapes. A small lamp with a white bulb illuminated the experimental tank. The light was directed beside the tank so that it did not shine directly on the cuttlefish but still provided enough light to produce clear behavioural video. Examples of sonograms and behavioural videos are available online as part of this article (supplementary material, Videos 1–3).

Outline of experimental procedure

Once moved from the home tank to the experimental tank, the cuttlefish was allowed to acclimate to the experimental tank for at least 2 h while we quantified behaviour, ventilation rate and heart rate. Then it was exposed once only to the startling visual stimulus (bird cut-out, see below). Behaviour, posture, ventilation rate and heart rate were quantified before and after the exposure. Several trials were performed on each cuttlefish. Trials on a given cuttlefish were separated by at least 2 days.

Quantification of behaviour, ventilation rate and heart rate during acclimatization

Fish stressed by subcutaneous injection of 2% or 3% formalin have elevated 'resting' heart rates and show less cardiac inhibition during their alternate response than control fish (Ide and Hoffmann, 2002). Similarly, hermit crabs that

have elevated 'resting' heart rates after surgery have diminished alternate responses (Cuadras, 1980). We do not know whether stressed cuttlefish, defined as having elevated heart rates, also show less cardiac inhibition during the alternate response. To verify that the behaviour, ventilation rate and heart rate of cuttlefish in the experimental tank decreased to a resting plateau, we monitored these parameters during the 2-h acclimatization. Behaviour was assessed for 30 s at each of the following times during acclimatization: 30, 45, 60, 75, 90, 105 and 120 min. A behaviour score of 1 denoted that the cuttlefish was sitting on the bottom of the tank for the entire 30-s assessment. A behaviour score of 2 denoted that the cuttlefish was moving using its fins for part of the assessment period. A behaviour score of 3 denoted that the cuttlefish was moving using jets for part of the assessment period. Ultrasound could only be performed on stationary cuttlefish. After each behavioural assessment, we estimated the ventilation rate and the heart rate of the cuttlefish using ultrasound. Heart rate was estimated by counting the number of full contraction cycles of the ventricle completed in 10 s. Ventilation rate was estimated by counting the number of full sweeps of the collar flaps completed in 10 s. We used the collar flaps instead of the mantle to determine ventilation rate because the collar flaps move visibly even during resting ventilation (Bone et al., 1994). If measurements of physiological rates were not successful within 5 min, the attempt was stopped. Measurements were always successful at 120 min.

To assess whether a resting plateau was achieved, we used the behaviour score, ventilation rate and heart rate for each time point measured during the 2-h acclimation period. Several trials were performed on the same cuttlefish. For behaviour, we first calculated the median behaviour score over all trials for each cuttlefish and for each time point. Then we calculated one median value over all cuttlefish for each time point. For ventilation rate and heart rate, we first calculated the rate difference between subsequent readings because absolute rates varied between cuttlefish and could not be meaningfully compared. These differences were then treated like the behaviour scores, resulting in one median value for all cuttlefish for each time point. A score of zero for ventilation rate or heart rate indicated no change from the last time point, and therefore that the rate was stable. The median behaviour

score, ventilation score and heart rate score were plotted and inspected for a plateau.

Introduction of the startling visual stimulus

At least 2 h after transfer to the experimental tank, we exposed the cuttlefish to a sudden visual stimulus. The stimulus was a white, bird-shaped, Styrofoam cut-out. The stimulus was moved at approximately 0.7 m s^{-1} over the experimental tank by a pulley system and left over the tank without further manipulation until the end of the trial. The trial ended when the cuttlefish settled on the bottom of the tank, and its ventilation rate and heart rate returned to approximately resting values (estimated over 10 s as in the previous section). The stimulus was bird-shaped because birds are putative predators of cuttlefish in the wild (Blaber and Wassenberg, 1989; Lipinski and Jackson, 1989). However, the shape of the stimulus is probably unimportant; we and previous researchers have noticed that cuttlefish and octopods perform the Deimatic Display to a variety of suddenly approaching objects, including rectangles (Johansen and Martin, 1962; Wells, 1979; Chichery, 1980; Wells, 1980).

Quantification of ventilation rate and heart rate before and after introduction of the startling visual stimulus

We used ultrasound to visualize either the movements of the collar flaps (ventilation rate) or ventricular contractions (heart rate) from at least 30 s before the stimulus until the cuttlefish started swimming after the stimulus. When visualizing the collar flaps, we could also see the contractions of the anterior vena cava, and when visualizing the ventricle, we could also see the contractions of the lateral venae cavae (veins named according to King et al., 2005). Contractions of the anterior vena cava and the lateral venae cavae are indicators of venous return. It was not possible to reliably image the ventricle and the collar flaps simultaneously. Therefore, there was no way to compare the ventilatory and cardiac responses of a cuttlefish in a given trial because only one could be imaged at a time.

If the cuttlefish moved during stimulus presentation, thereby preventing continuous data collection with the ultrasound, the trial was discarded (10/26 trials). We performed enough separate trials (each including acclimatization and one presentation of the stimulus) on each cuttlefish to obtain at least one example of its ventilatory response and one example of its cardiac response to sudden stimuli. Data were successfully collected from the collar flaps (ventilation) in one trial for each of the six cuttlefish. Systemic heart data were successfully collected for all cuttlefish, three cuttlefish producing more than one successful trial (10 trials total).

During video playback after the trial had ended, we determined ventilation and heart rates. These rate calculations were separate from and more precise than the estimates of ventilation rate and heart rate made during acclimatization (see above section on acclimatization). They are presented as per minute (min^{-1}) rates in the results (*vs* 10 s rates presented for acclimatization). From successful sonograms of the collar flaps, we recorded the times (±1/15 s) at which the collar flaps

completed each full ventilation cycle. In successful sonograms of the systemic heart, we recorded the times (±1/15 s) at which the ventricle was fully contracted. For both the collar flaps and the ventricle, we used the time it took to complete each cycle to calculate the rate min^{-1} using the equation: rate min^{-1}=(period of contraction)$^{-1}$×60, where the period of contraction is measured in s.

These min^{-1} rates were plotted and inspected using recent procedures of visual inspection (Ide and Hoffmann, 2002; Cooke et al., 2003). Individual min^{-1} rates after the stimulus had to differ from <u>all</u> individual min^{-1} rates before the stimulus by at least the measurement error in order to be considered a true change. The measurement error was taken as 1/15 s, because this was the accuracy of our beat-to-beat time measurements (see above). Explicitly stated, we found the lowest min^{-1} rate before the stimulus and lowered it by the measurement error (±1/15 s). If any of the 7 rates immediately following the stimulus were lower than this, the rate was considered to have decreased. Similarly, to determine whether the rate rose above resting values after the stimulus, we found the highest rate before the stimulus and increased it by the measurement error (±1/15 s). If any of the seven rates after the stimulus were higher than this, the rate was considered to have increased. Sometimes, the rates both decreased below and increased above resting levels after the stimulus in the same trial. As a baseline for each trial, we averaged together the min^{-1} rates for the entire 30-s resting period before the stimulus. The magnitude of increases or decreases in rates was calculated as a percentage of the baseline rate.

Quantification of the behavioural and postural responses to the startling visual stimulus

Behavioural data were obtained for all 26 trials, and displayed considerable variability. The behavioural reaction during the alternate response was best described as the Deimatic Display (e.g. Fig. 1). Variability in the Deimatic Display has been noted by previous authors (Chichery, 1980; Hanlon and Messenger, 1996), who also identified its key components. In order to quantify the chromatic and textural reaction of the cuttlefish to the stimulus, we selected the four key chromatic and textural components of the display that differed obviously between our trials: Mantle paling, paired Mantle spots (that look like eyes), dark elements on the fins, and Smooth mantle (i.e. mantle devoid of papillae). The number of these that occurred during a given trial was called the chromatic and textural index.

Mantle flattening and Arm spreading are two key postural components of the Deimatic Display. Mantle flattening implies both a lateral widening of the mantle and a reduction in its dorso-ventral height. To quantify Mantle flattening and Arm spreading, we used the public domain NIH Image program (version 1.62, developed at the US National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). We measured the mantle and arms at the points described below and depicted in Fig. 2, before and after the stimulus.

A

Fig. 2. The parameters measured to quantify posture. (A) The width of the mantle was measured along line a, the width of the arms along line b. (B) Schematics of the cross-sectional view through plane 1 in A (sonogram of collar flaps). The left hand schematic represents an animal at rest, the right, an animal during the alternate response. The dorso-ventral height of the anterior mantle was measured along line c. The area of the anterior mantle cavity (water space) was stippled area d. (C) Schematics of the cross-sectional view through plane 2 in A (sonogram of the ventricle). As in B, the left hand schematic represents an animal at rest, the right, an animal during the alternate response. The dorso-ventral height of the mid-mantle was measured along line e. The area of the mid-mantle cavity was stippled area (f). avc, anterior vena cava; cf, collar flap; fu, funnel; m, mantle; all grey areas represent viscera; all white areas represent fluid-filled spaces.
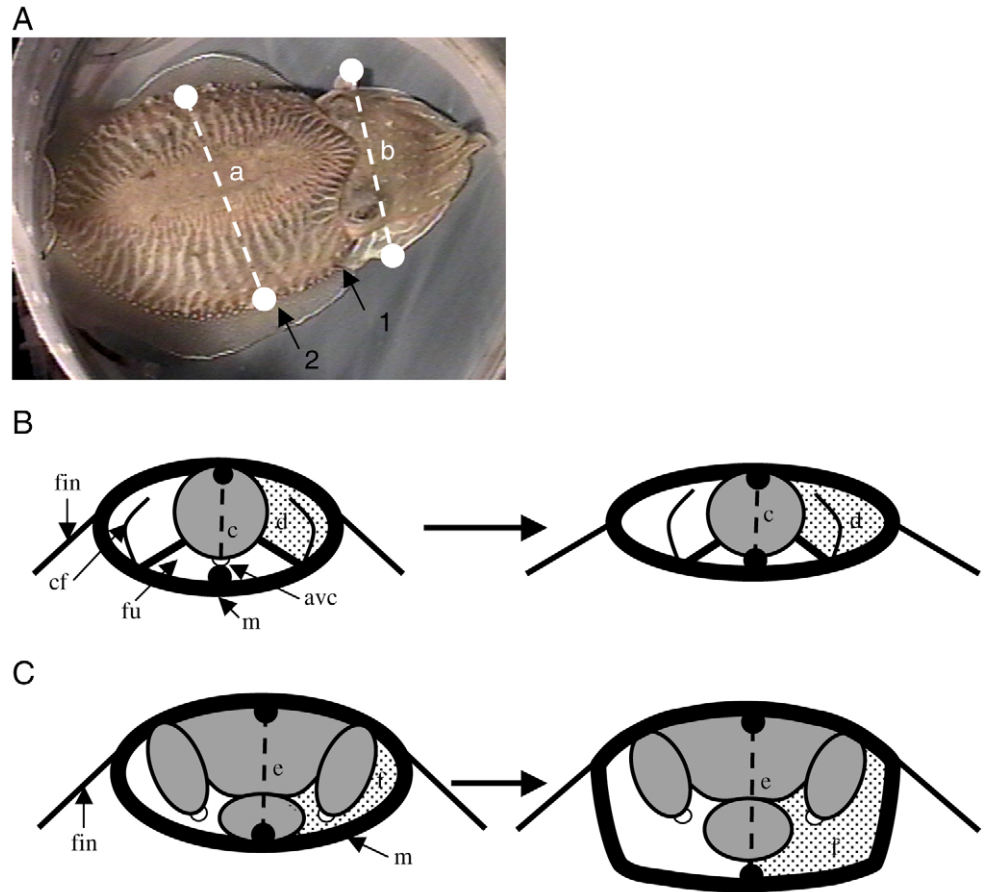
From the behavioural video: (a) the width of the mantle approximately halfway down the mantle (the 'mid-mantle'); (b) the distance between the outer edges of the base of the 4th arms.

From the sonogram of the collar flaps: (c) the distance between the cuttlebone and the inside surface of the mantle (dorso-ventral height of the anterior mantle); (d) the area of the water space in the anterior mantle cavity (one side only).

From the sonogram of the ventricle: (e) the distance between the cuttlebone and the inside surface of the mantle (dorso-ventral height of the mid-mantle); (f) the area of the water space of the mid-mantle cavity (one side, from the midline).

For each trial, we captured one frame on an inhalation 5 s or less before the stimulus (resting) and another frame after the stimulus at the onset of the most intense part of the behavioural reaction. The resting frame and the reaction frame were each measured three times. The mean of the three measurements was the reported value. The standard deviation (s.d.) of the three measurements was the measurement error. If the mean measurement before and after the stimulus did not differ by more than the measurement error, we considered there to be no change. We omitted trials in which the cuttlefish changed orientation after the stimulus. If we measured the change in one of the parameters in more than one trial for a given cuttlefish, the mean change was reported for that cuttlefish.

Summary statistics describe the median and quartiles of these means over all animals. Measurements before and after stimulus presentation were compared using a paired-sample *t* test (Zar, 1999) because sizes are likely to originate from a normally distributed population. We did not report on the following key components of the Deimatic Display: Fin stalling (i.e. the fins stop moving) and spreading, Pupil dilation and Dark ring around the eye. The last two were seldom seen in our experiments.

Results

Behaviour, ventilation rate and heart rate during acclimatization

Immediately after transfer, cuttlefish swam agitatedly around the experimental tank. However, from min 75 of the acclimatization onward, cuttlefish showed a median behaviour score of 1. By 90 min, the third quartile also fell to 1 and remained at 1 for the rest of the acclimatization, indicating that cuttlefish were resting quietly on the bottom of the experimental tank (Fig. 3A).

During acclimatization, the median ventilation rate did not drop between the first reading at 30 min and the last reading at 120 min (Fig. 3B). Median heart rate decreased until 90 min (as indicated by negative values), and then became stable (Fig. 3C).
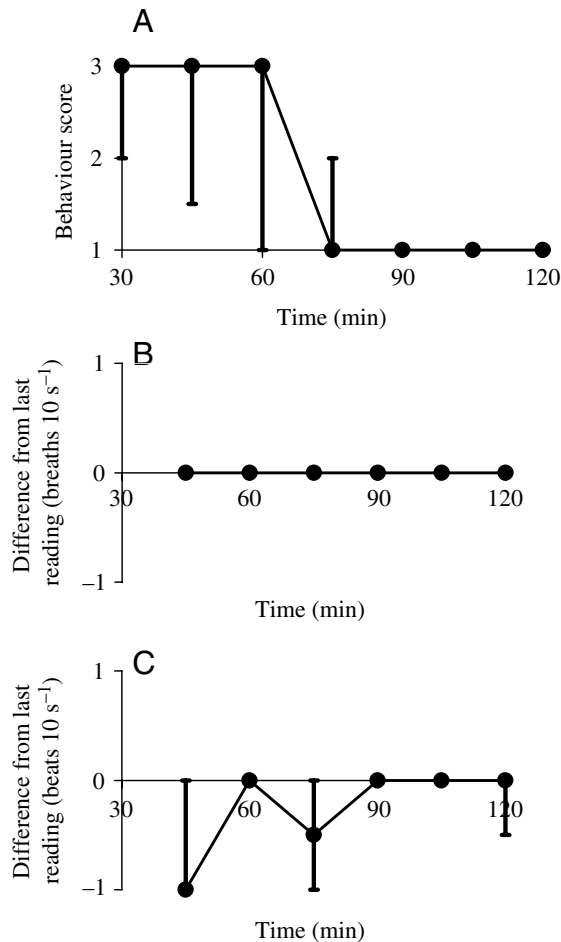
Fig. 3. (A) The median behaviour scores (±1st and 3rd quartiles) during the 2-h acclimatization period over all cuttlefish (*N*=6). A score of 1 indicates resting cuttlefish. (B) Median difference between subsequent estimations of ventilation rate (±1st and 3rd quartiles) during the 2 h acclimatization period. A score of 0 indicates no change between readings; a negative score indicates falling rates (*N*=6). (C) Same as B, but for heart rate (*N*=6).
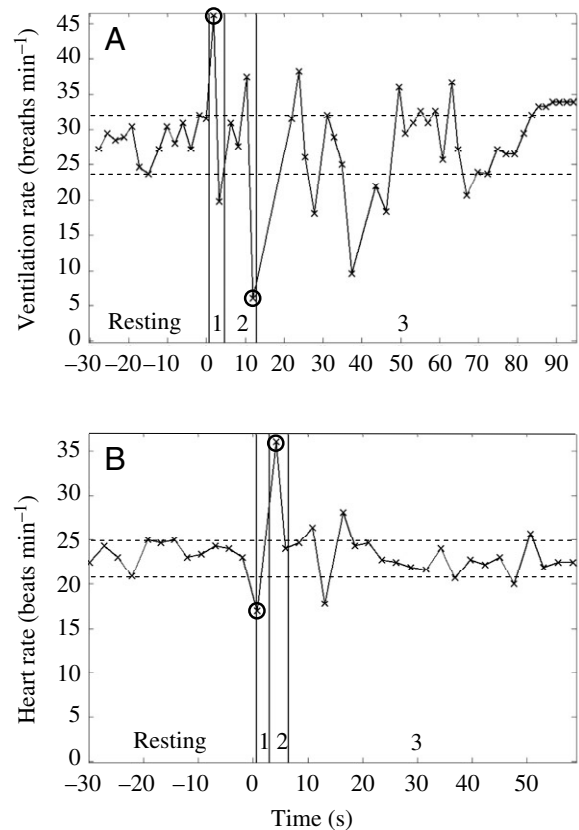

Fig. 4. Representative examples of the ventilatory (A) and cardiac (B) reactions to startling stimuli. The stimulus was presented at time 0. Vertical lines delineate resting behaviours and reaction stages 1–3. Broken lines delimit the maximum and minimum rates before the stimulus. Circles emphasize maximum and minimum rates after the stimulus. (A) Cuttlefish #12: decrease in ventilation rate: 78.9% (Video 2 in supplementary material). (B) Cuttlefish #22: decrease in heart rate: 28.3%.

The four locomotory stages of the alternate reaction

After 120 min, the cuttlefish were exposed to a sudden visual stimulus. Observations from the behavioural video indicated that cuttlefish typically had a four-stage locomotory reaction to the stimulus (e.g. Video 1 in supplementary material). These stages are summarized in Table 3, and described below.

The first and shortest stage (duration range: 1–12 s) started at the onset of the behavioural response. It consisted primarily of subtle movements such as slowing of fin undulations and widening of the arms and mantle. During the second stage, the cuttlefish showed behavioural freezing for a period of 1–34 s. No movement of the mantle, collar flaps, fins or arms were visible from above during this stage. The third stage started when movement again became discernible (duration range of stage 3: 7–332 s). This usually started with ventilation and the resulting movements of the mantle, fins and the lateral ridges on the fourth arms. Movements usually became progressively larger until the cuttlefish started swimming at the start of the

fourth stage. The fourth stage ended when the cuttlefish again settled on the bottom of the experimental tank and its heart and ventilation rates returned approximately to resting values. The fourth stage was the longest and took between 3 and 28 min. All six cuttlefish typically showed this four-stage pattern, although four of the six cuttlefish occasionally skipped stages (6/26 trials). Two did not become immobile in some of their trials (cuttlefish #11 skipped stage 2 in 2/6 trials, #12 in 2/4 trials). Two cuttlefish did not swim in some of their trials (cuttlefish #17 and #22 skipped stage 4 in 1/4 trials each).

Ventilatory and cardiac reactions to sudden visual stimuli

From the sonograms that were recorded simultaneously with the behaviour video, we quantified how ventilation rate (Video 2 in supplementary material) and heart rate (Video 3 in supplementary material) changed after presentation of the visual stimulus. We also monitored venous contractions of the anterior vena cava and lateral venae cavae on the sonograms. Because sonograms and behavioural video recordings were synchronized by an audio cue, we were able to determine when

Table 3. *Summary of the four locomotory stages of the alternate reaction in cuttlefish*

	Stage 1	Stage 2	Stage 3	Stage 4	End of experiment
Defining locomotion	Slowing of movements	Behavioural freezing	Resumption of movement	Swimming	Resumption of resting behaviour
Duration	3 s (2,5 s)	3 s (3,12 s)	40 s (13,77 s)	9 min (7,16 min)	N/A
Important ventilatory and cardiac events (see Table 4)	Heart rate decreased below resting levels	Ventilation rate decreased below resting levels	Ventilation rate and heart rate recovered	Rates impossible to measure due to swimming	Ventilation rate and heart rate returned to resting
Important chromatic and textural events (see text)	Rapid chromatic and textural changes	Some textural changes; Deimatic Display	Recovery from Deimatic Display	Recovery from Deimatic Display	None

Duration is expressed as the median over all cuttlefish ($N=6$; 1st, 3rd quartiles in parentheses). Also included are the important ventilation rate, heart rate and chromatic and textural changes.

the changes in ventilation rate and heart rate occurred relative to the four locomotory stages described in the previous section and in Table 3.

Before presentation of the stimulus, the mean ventilation rate for all six cuttlefish was 35.0±9.3 breaths min^{-1} (calculated from baseline, not acclimatization, rates; see Materials and methods). After presentation of the stimulus, ventilation rate always decreased below baseline rates, either in stage 2 or at the onset of stage 3 (range: 24.8–84.6%; Table 4). Ventilatory movements of the collar flaps not only slowed, but stopped entirely in 4/6 cuttlefish for 3.7–16.3 s. The % decrease in the ventilation rate was inversely proportional to the baseline ventilation rate of the cuttlefish (linear regression: $r^2=0.95$, $P=0.0009$, $N=6$). In other words, the lower the rate before the stimulus, the larger the % drop in rate after the stimulus. Animals with lower baseline rates were also more likely to experience ventilatory arrest. Before decreasing, ventilation rate increased above baseline for one breath in 3/6 cuttlefish (e.g. Fig. 4A). These were not always the trials that subsequently showed ventilatory arrest.

In resting cuttlefish, the contractions of the anterior vena cava have the same rate as ventilatory contractions (King et al., 2005). After presentation of the sudden stimulus, the contractions of the anterior vena cava slowed or stopped almost simultaneously with the movements of the collar flaps (4/5 cuttlefish where contractions of the anterior vena cava were visible).

Before presentation of the stimulus, the mean heart rate for the six cuttlefish was 24.0±5.1 beats min^{-1} (calculated from

baseline, not acclimatization, rates; see Materials and methods). If all reaction stages were present, heart rate decreased after presentation of the sudden stimulus, typically in stage 1 (range: 11.6–82.3%; Table 4). If cuttlefish did not show all stages of the reaction, then there was either no decrease in heart rate (2/3 trials, 2/3 cuttlefish), or a very modest one (12.1% decrease, 1/3 trials, 1/3 cuttlefish). Cardiac arrest occurred in 3/7 trials (2/5 cuttlefish; these trials also included all reaction stages) and lasted for 5.3–20.0 s. As with ventilation rate, the percentage decrease in heart rate was inversely proportional to the baseline rate of the cuttlefish (linear regression: $r^2=0.83$, $P=0.03$, $N=5$), and animals with the lowest baseline rates were more likely to experience cardiac arrest. Heart rate occasionally increased above baseline (2/7 trials, 2/7 cuttlefish). In contrast to increases in ventilation rate, increases in heart rate occurred for one contraction immediately <u>after</u> the lowest heart rate (Fig. 4B).

In resting cuttlefish, contractions of the lateral venae cavae have the same contraction rate as the systemic heart (King et al., 2005). After the sudden stimulus, the contractions of the lateral venae cavae slowed or stopped almost simultaneously with those of the systemic heart (9/10 trials, 6/6 cuttlefish).

Chromatic and textural reactions to sudden stimuli

In the 30-s period before the stimulus, cuttlefish showed resting body colouration (King, 2005). Upon presentation of the stimulus, 5/6 cuttlefish showed a chromatic and textural reaction consistent with the Deimatic Display (e.g. Video 1 in

Table 4. *Summary of the ventilatory and cardiac responses of cuttlefish to sudden stimuli*

	No. of trials No. of cuttlefish	% Rate decrease median (1st, 3rd quartiles)	Stage that decrease occurred	Occurrence of arrest	Duration of arrest, range (s)
Ventilation rate	6	63.5	Stage 2: 4/6 cuttlefish	4/6 trials	3.7–16.3
	6	(46.0, 78.9)	Onset of stage 3: 2/6 cuttlefish		
Heart rate	7	48.3	Stage 1: 4/5 cuttlefish, 5/5 trials	3/7 trials	5.3–20.0
	5	(20.0, 66.5)	Stage 2: 1 cuttlefish, 2/2 trials	2/5 cuttlefish	

Only trials having all reaction stages are included. All trials showed ventilatory or cardiac slowing, but not all showed arrest.

supplementary material). The individual components of the Deimatic Display varied in duration and intensity both among trials on the same animal and among animals. Only one cuttlefish, #22, showed a chromatic response that was inconsistent with the Deimatic Display (he darkened in 2/4 trials). This cuttlefish also showed unusual postural responses (see next section).

To place the chromatic and textural reaction in a temporal framework, we investigated the stages at which different components of the chromatic and textural index appeared. Mantle spots (7/8 occurrences), Mantle paling (14/16 occurrences), and dark elements on the fins (18/19 occurrences) usually started in stage 1 of the reaction. Mantle smoothing started in stage 1 (2/5 occurrences) or stage 2 (3/5 occurrences). The different components often faded slowly, making end points difficult to determine. They started fading as early as stage 2 or as late as stage 4 (Table 3).

To assess the connection between the chromatic reactions and the ventilatory and cardiac reactions, we tested whether the chromatic and textural index was correlated to the same things as the physiological reactions, i.e. the presence of all reaction stages and the resting physiological rates. The chromatic and textural index ranged from 1–4 both when cuttlefish showed all reaction stages (20 trials) and when they skipped reaction stages (6 trials), i.e. the most intense chromatic and textural reactions were possible even when reaction stages were missing. When the average chromatic and textural index for an animal was compared to its average resting ventilation rate and its average resting heart rate, there was no correlation (linear regression: *vs* ventilation rate r^2=0.025, *P*=0.76, *N*=6; *vs* heart rate r^2=0.030, *P*=0.74, *N*=6). Unlike physiological responses to sudden stimuli, chromatic and textural responses were not tied to resting physiological rates.

Postural responses to sudden visual stimuli

Besides changing their locomotion, ventilation and heart rates, and skin colour and texture, cuttlefish also changed their shape after seeing a sudden stimulus. We quantified the shape change of the anterior mantle (e.g. Video 2 in supplementary material) and the mid-mantle (e.g. Video 3 in supplementary material) and compared it to the other aspects of the reaction described above.

Measurements from the overhead behavioural video revealed that the mid-mantle and the arms both widened significantly during the cuttlefish alternate response (Table 5). Measurement of anterior mantle depth was possible only from the sonograms of 4/6 cuttlefish. In these, the anterior mantle always became shallower after the stimulus (4/4 cuttlefish, Table 5). In 3/6 cuttlefish, we were able to measure the cross-sectional area of the mantle cavity (water space) beside the neck. This always increased (3/3 cuttlefish, Table 5). Therefore, decreasing mantle depth resulted in larger, not smaller, anterior mantle water spaces. Measurements from the sonograms of the systemic heart unexpectedly revealed that the mid-mantle sometimes became deeper after the stimulus (4/6 cuttlefish; range: 3.6–37.9%). In the other two cuttlefish, it became modestly shallower (–5.7% and –6.3%). We could measure the area of the mid-mantle water space in 5/6 cuttlefish. This increased, often dramatically, in 4/5 cuttlefish (Table 5). In a fifth cuttlefish, #22, it stayed the same. This fifth cuttlefish, however, also showed the unusual chromatic responses (Mantle darkening) discussed in the last section. Given the high variability in area increases (range: 0–175.3%) and the small sample size (*N*=5), the increase had only borderline significance (*t*=2.2, t_{crit}=2.8, 0.05<*P*<0.10).

Overall, the change in mantle shape drew water into the anterior and mid-mantle. To facilitate discussion, the increase in mantle water volume will be called hyperinflation; the cross-sectional areas measured were larger than the mantle inflation seen during normal ventilation. To understand the role that changes in mantle shape might play in the alternate reaction, we compared the degree of hyperinflation to other aspects of the response (decreases in heart rate, the chromatic and textural index and the reaction stages). Percentage hyperinflation of the mid-mantle was chosen because it was the most available descriptor of mantle shape change.

Hyperinflation and decreased heart rate could be related. The magnitude of hyperinflation was related to % decrease in heart rate, with borderline significance (linear regression: r^2=0.74, *P*=0.062, *N*=5; Fig. 5). Furthermore, maximum hyperinflation typically occurred when heart rates were falling or at their minimum (6/8 trials, 4/5 cuttlefish; however cuttlefish #26 showed maximum hyperinflation before any response in heart rate in 2/2 trials). Similar to the magnitude of cardiac

Table 5. *The percentage change in postural parameters measured before and after presentation of the stimulus*

Postural parameter	Median % change (1st, 3rd quartile)	N	Paired-sample *t* test	
			t	P
(a) Mid-mantle width	11.3 (10.1,12.6)	6	6.92	<0.001
(b) Arm width	15.6 (11.5,19.6)	6	5.89	<0.005
(c) Anterior mantle depth	−18.5 (−17.1,−20.4)	4	16.67	<0.001
(d) Anterior mantle cavity area	8.7 (8.3,15.6)	3	4.58	<0.05
(e) Mid-mantle depth	4.9 (−5.7,12.5)	6	1.20	NS
(f) Mid-mantle cavity area	70.7 (3.2,164.0)	5	2.24	<0.10

See Fig. 2 for an explanation of measured parameters.
Measurements that were larger after the presentation are positive; those that were smaller are negative.
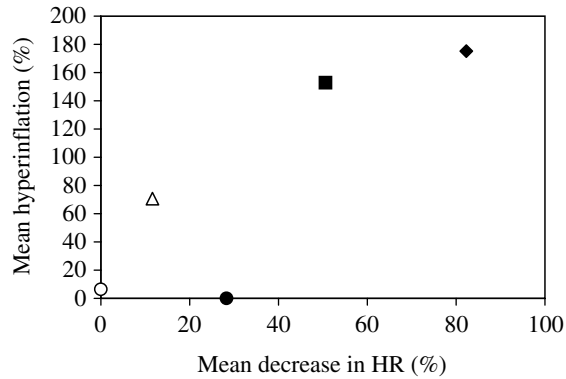
Fig. 5. The relationship between the % decrease in heart rate (HR) and % hyperinflation. Each point represents the averaged data of one animal. Open symbols indicate animals that missed reaction stages in 50% or more of trials, closed symbols indicate animals that showed all reaction stages in more than 50% of trials. The average chromatic index is represented by the shape of the symbol: circle=1, diamond=2, triangle=3, square=4. The outlying closed circle represents cuttlefish #22, who showed different chromatic (Mantle darkening) and postural responses from the other cuttlefish.

decreases, the magnitude of hyperinflation was greatest in trials with all reaction stages (Fig. 5).

The magnitude of the chromatic and textural index was not correlated to the magnitude of mid-mantle hyperinflation (linear regression: r^2=0.53, P=0.16, N=5; Fig. 5). Therefore, we have no evidence that chromatic and textural changes were related to locomotory changes, to ventilatory and cardiac changes, or to postural changes.

Discussion

Similar to several vertebrates and invertebrates (see Table 1 and introduction for references), cuttlefish decreased their ventilation rate and heart rate and exhibited behavioural freezing in response to sudden stimuli. Unlike other animals, cuttlefish also changed the colour and texture of their skin (elements of the Deimatic Display) during the response. Interestingly, the number of measured chromatic and textural changes was not related to any other measured parameter. This is perhaps not surprising considering that the Deimatic Display is believed to signal to a potential predator that it has been seen (Hanlon and Messenger, 1996). Such anti-predation signals may need to be sent regardless of the cuttlefish's ventilation rate, heart rate or posture. The intensity of the Deimatic Display is probably more dependant on what is appropriate to the external environment than to the internal environment of the cuttlefish.

In contrast, the % decreases in both ventilation rate and heart rate were inversely proportional to their resting rates; lower resting rates were associated with a larger percentage decrease in rate, while higher resting rates were associated with a smaller percentage decrease. This may seem counterintuitive because higher rates should have 'further to fall' during the response, by the Law of Initial Values (Richards, 1980). However, this phenomenon is not restricted to cuttlefish. Fish

(Ide and Hoffmann, 2002) and crabs (Cuadras, 1980) with elevated heart rates also show reduced drops in heart rate during the alternate response. Cuttlefish with high resting rates may have had high resting metabolic demands (e.g. during digestion, pathology or oogenesis) that necessitated continuous, elevated oxygen distribution. This, in turn, could have prohibited any decreases in heart rate or ventilation rate.

It is important to note that our results were obtained from resting cuttlefish that had been allowed to acclimatize for at least 2 h. It is possible that a longer acclimatization could have resulted in lower resting rates and in larger drops in ventilation and heart rates after the stimulus. By the same token, it is possible that non-resting cuttlefish (e.g. swimming, exploring, hunting) may have had lesser or different responses than the ones we observed. For example, they might have had a jetting response (fight or flight) instead of an alternate response.

Given the inverse relationship between the baseline rate and the magnitude of the ventilatory and cardiac reaction, it is important to use non-invasive techniques to study changes in the ventilatory and cardiac function during the alternate reaction in cephalopods. Our trials using ultrasound resulted in lower resting ventilation rates and heart rates than did experiments using some invasive techniques (Table 6). It is possible that we observed decreased heart rate during the alternate reaction while Chichery (Chichery, 1980) saw no change because his cuttlefish showed elevated resting heart rates (45–50 beats min^{-1} at 13°C *vs* our 24.0±5.1 beats min^{-1} at 15°C). Unfortunately, the size and number of cuttlefish he used are not reported. Given the inverse relationship between size and heart rate (Chichery and Chanelet, 1972), it is possible that his animals were simply smaller than ours, rather than more stressed.

Ultrasound imaging was also useful because it allowed us to study many aspects of the alternate response simultaneously, including the reactions of the contractile veins. It is interesting that all venous contractions, as well as cardiac contractions, slowed or stopped during the cuttlefish alternate response. In cuttlefish, blood is returned to the systemic heart by the active contraction of the veins (King et al., 2005). Consequently, the heart might not be able to stop pumping unless the contractions of the upstream veins are also inhibited. More powerful ultrasound equipment (e.g. equipped with Doppler) will allow future studies to determine whether cardiac arrest resulted in circulatory standstill. Furthermore, as multiple aspects of the alternate response are studied simultaneously and across animal groups using non-invasive techniques, it will become apparent whether attributes other than decreased ventilation and heart rate are shared between animal groups. This might help us elucidate the function, if any, of this response.

In cuttlefish, it is unlikely that ventilation rate and heart rate decreased to increase crypsis (hypothesis 1, Table 2). Ventilation rate and heart rate decreased during different stages. Therefore, either the mantle or the heart was always a potential source of movement and noise. Furthermore, the decreases were accompanied by the sudden and obvious chromatic changes of the Deimatic Display. It is similarly unlikely that decreased ventilation rate and heart rate were due

Table 6. *Resting ventilation and heart rates reported in this and previous studies on S. officinalis*

Study	Technique	Temperature (°C)	Cuttlefish size (mantle length, cm)	Reported rate	Standardized rate
Ventilation rate (breaths min^{-1})					
Present study	Non-invasive (ultrasound)	15	15.5–18.5	35.0±9.2	35.0±9.2
Boal and Ni, 1996	Non-invasive (visual observation)	19–21	7.5–10.5	45	32.1
Mislin, 1966	Invasive (leads forced through mantle)	16–18	20–35	45±9	39.3
Bone et al., 1994	Invasive (electrodes in mantle tissue)	18.5	5.0–25.0	48–60	42.6
Heart rate (beats min^{-1})					
Present study	Non-invasive (ultrasound)	15	15.5–18.5	24.0±5.1	24.0±5.1
Chichery and Chanelet, 1972	Invasive (EKG leads)	16	13.6–20.0	20–30	22.8
Mislin, 1966	Invasive (leads forced through cuttlebone)	16–18	20–35	43±9	35.8
Chichery, 1980	Invasive (EKG leads)	13	Not reported	45–50	57.1

Rates are standardized to our temperature of 15°C using previously calculated Q_{10} values (King et al., 2005). If size was reported in mass instead of in mantle length, the equivalent mantle length was interpolated (Forsythe et al., 1994). We did not standardize rates for size. However, larger animals are expected to have slower heart rates (Chichery and Chanelet, 1972).

to decreased metabolic rate during the cuttlefish alternate reaction (hypothesis 2, Table 2). In fact, electromyograms reveal that cuttlefish mantle muscle and head retractor muscles start a continuous and vigorous contraction during the alternate response (Chichery, 1980). This very likely increases metabolic demand during the response.

The most likely adaptive function of respiratory arrest is the need to prepare for flight. During the alternate response, cuttlefish almost always stopped mantle movements during hyperinflation, i.e. held their mantles full of more water than during normal ventilation. Chichery (Chichery, 1980) found similar results. The extra water held in the mantle during hyperinflation and subsequent immobility would be useful for jetting, should the cuttlefish then decide that it needed to flee. Jetting can follow the Deimatic Display if the stimulus (or predator) continues to approach (Hanlon and Messenger, 1996). Every jetting cycle is started by hyperinflation (Packard and Trueman, 1974), and if cuttlefish filled their mantles in advance, they could jet immediately when chromatic displays failed to deter a predator.

A disadvantage of sustained hyperinflation is that it requires the cuttlefish to 'hold its breath' and might also require cardiac inhibition. Not only did maximum hyperinflation coincide with falling or minimum heart rates, but trials with large amounts of hyperinflation also had large decreases in heart rate. During hyperinflation, the mantle expands and thins (Packard and Trueman, 1974) through contraction of the radial mantle muscles (Bone et al., 1994). It is possible that, similar to mammals (Guyton, 1991), muscle contraction compresses intramuscular blood spaces. In cuttlefish, most capillaries run perpendicular to the radial muscles (Bone et al., 1981), and therefore would be compressed by radial muscle contraction. During intense radial muscle contraction, as is seen during hyperinflation (Bone et al., 1994), capillaries may be so compressed that blood flow through the mantle is almost stopped. If cardiac output remained constant, all pumped blood would be forced into the head and viscera of the cuttlefish, resulting in undesirable increases in intravascular pressure in those areas.

Further evidence supports the theory that small blood vessels in the mantle collapse during hyperinflation. First, our cuttlefish's anterior venae cavae and systemic hearts filled despite cardiac arrest, possibly because venous blood was being forced into them from the compressed intra-mantle blood spaces. Second, aortic blood pressures do not drop, but increase during cardiac arrest in the octopus *Enteroctopus dofleini* Wülker, indicating large increases in peripheral resistance during the alternate response (Johansen and Martin, 1962). Third, whenever octopods jet, even in the absence of startling stimuli, their hearts always stop (Johansen and Martin, 1962; Wells et al., 1987). Jetting requires strong muscular contraction of the mantle (Bone et al., 1994). Conversely, when an octopus moves using its arms, which does not require strong mantle contractions, heart rate increases rather than decreases (Wells et al., 1987). This suggests that movement alone does not necessitate a drop in heart rate, but instead that cardiac function appears to be interrupted by contractions of the radial muscles. Interrupted cardiac function is not seen in jetting squid (Wells et al., 1988; Shadwick et al., 1990). However,

squid jet constantly and may prevent blood from being forced into the rest of the body during jetting by using muscular valves ('peripheral hearts', Williams, 1909). 'Peripheral hearts' have not been found in *Sepia* or in *Octopus*, who rely primarily on their fins and arms, rather than their mantles, for routine locomotion.

To conclude, we hypothesize that the decrease in ventilation rate and heart rate seen in cuttlefish after a sudden stimulus is a by-product of the cuttlefish's preparation for flight. While the exact mechanism in cuttlefish involves species-specific peculiarities of its circulatory system, decreased heart rate in other animals may also be due to increases in peripheral resistance. Large increases in peripheral resistance occur during the typical mammalian stress response (Guyton, 1991). Increased activity of all appendages occurs during the alternate response of crabs (Cuadras, 1980), potentially resulting in increased peripheral resistance. If decreased heart rate after a sudden visual stimulus is the result of increased peripheral resistance, it suggests that decreased heart rate may be a necessary and common part of the stress response, but it might not itself be adaptive.

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