The Journal of Experimental Biology 208, 2071-2082
Published by The Company of Biologists 2005
doi:10.1242/jeb.01575



# Using ultrasound to understand vascular and mantle contributions to venous return in the cephalopod *Sepia officinalis* L.

Alison J. King[1,*], Stephen M. Henderson[4], Matthias H. Schmidt[2], Alison G. Cole[1,†] and Shelley A. Adamo[3]

[1]*Department of Biology,* [2]*Department of Radiology* and [3]*Department of Psychology, Dalhousie University, Halifax, NS, Canada* and [4]*Scripps Institution of Oceanography, La Jolla, CA 92093-0209, USA*

*Author for correspondence (e-mail: ajking@dal.ca)

†Present address: School of Medicine, Department of Orthopedics and School of Veterinary Medicine, Department of Anatomy, Physiology and Cell Biology, University of California, Davis, CA 95616, USA

*Accepted 8 March 2005*

## Summary

**Using ultrasound imaging, we investigated the roles of the potentially contractile veins and of the mantle (the powerful body wall that moves water over the gills, and also encloses the large veins and the hearts) in returning the blood of cuttlefish to its hearts. Ultrasound provided the first non-invasive observations of vascular function in an unanaesthetized, free-moving cephalopod. The large veins (anterior vena cava, lateral venae cavae and efferent branchial vessels) contracted in live, intact cuttlefish (*Sepia officinalis* L.). The anterior vena cava contracted at the same rate as the mantle, but it often expanded during mantle contraction. Furthermore, the anterior vena cava contracted peristaltically *in vivo*, suggesting that it actively aids venous return. The lateral venae cavae and efferent branchial vessels contracted at the same rate as the branchial and systemic hearts, but at a different rate from the mantle. A peristaltic wave appeared to travel along the lateral venae cavae to the branchial hearts, potentially aiding venous return. We found a muscular valve between the anterior and lateral venae cavae, which ensured that blood flowed only one way between these unsynchronized vessels. The mantle appears to have an unclear connection with cardiovascular function. We conclude that, when cuttlefish are at rest, the mantle does not compress any of the large veins that we imaged (including the anterior vena cava), and that peristaltic contractions of the large veins might be important in returning cephalopod blood to the hearts.**

Supplementary material available online at
http://jeb.biologists.org/cgi/content/full/208/11/2071/DC1

Key words: cardiovascular dynamics, venous return, ventilation, cephalopoda, mantle, circulation, systemic heart, branchial heart, vein, cuttlefish, *Sepia officinalis.*

## Introduction

Millions of years of exposure to similar selective pressures have resulted in convergent evolution between modern coleoid cephalopods and modern chondrichthian and teleost fishes (Packard, 1972; O'Dor and Webber, 1986; Hanlon and Messenger, 1996). The cephalopod circulatory system was shaped by this convergent evolution (Packard, 1972; O'Dor and Webber, 1991; Wells, 1994; Hanlon and Messenger, 1996). Unlike other molluscs, which have open circulatory systems (Brusca and Brusca, 1990), modern coleoids (e.g. octopods, cuttlefish and squid) have high pressure (Wells, 1979; Bourne, 1982), high output (Shadwick et al., 1990), closed (Williams, 1909; Tompsett, 1939; Wells, 1978; Schipp, 1987a) circulatory systems resembling those of fishes (Farrell and Jones, 1992). Furthermore, coleoids have two separate circulations (Tompsett, 1939), one for the gills, powered by the single-chambered branchial hearts at the base of the each gill, and one for the systemic circulation, powered by the ventricle and its two auricles (Fig. 1). In this regard, the coleoid circulatory system appears more avian or even mammalian than fish-like (Packard, 1972).

Despite the many similarities between coleoid and vertebrate circulatory systems, coleoid systems are based on the molluscan *Bauplan*, and therefore differ in important ways from vertebrate systems. For example, some of the valves guarding the entrances and exits to coleoid hearts are muscular and innervated (Smith and Boyle, 1983). This is more reminiscent of crustacean (Wilkens, 1997; Davidson et al., 1998) than mammalian cardiovascular valves (Berne and Levy, 1997). Unlike vertebrates and crustaceans, many coleoid venous vessels contract *in vitro* (Smith and Boyle, 1983; Schipp, 1987a). Perhaps one of the most important factors affecting basic circulation in cephalopods is their oxygen transport protein, hemocyanin. It is not contained in blood cells, but instead is dispersed freely in the blood (Mangum,

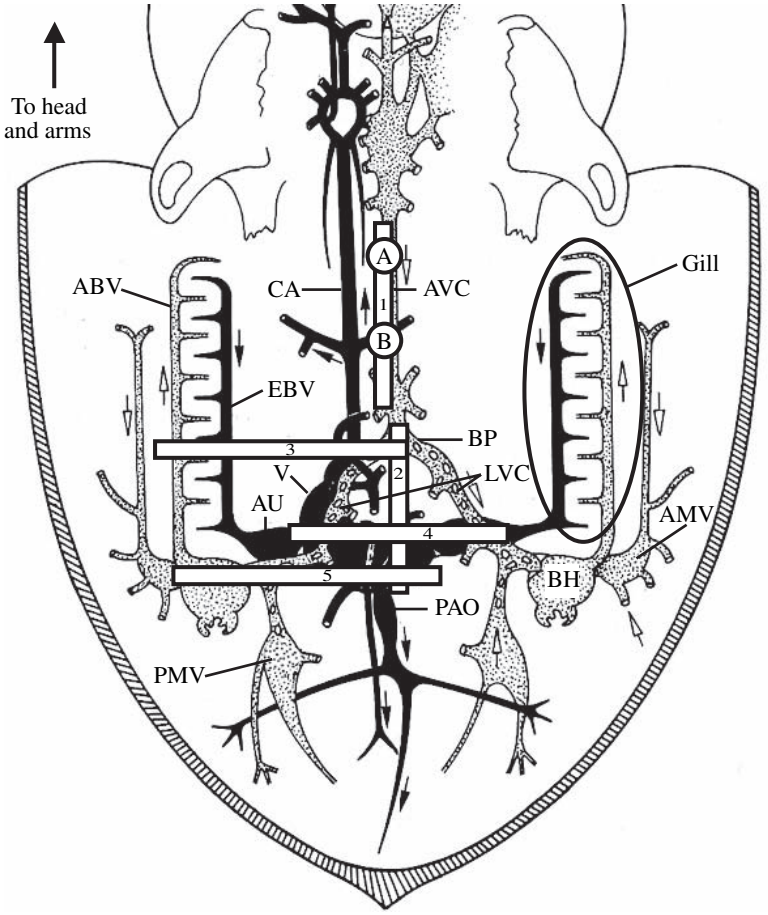


Fig. 1. The circulatory system of *S. officinalis* viewed from below (modified from Schipp, 1987b). White rectangles indicate planes along which ultrasound images were taken. See Materials and methods for organs transected in each numbered plane. Stippled vessels carry deoxygenated blood; dark vessels carry oxygenated blood. ABV, afferent branchial vessel; AMV, anterior mantle vein; AU, auricle; AVC, anterior vena cava (Point A, near the opening of the mantle; Point B, near the opening of the anus); BH, branchial heart; BP, branch point; CA, cephalic aorta; EBV, efferent branchial vessel; LVC, lateral venae cavae; PMV, posterior mantle vein; PAO, posterior aorta; V, ventricle.

1990; Pörtner, 1994). Despite having the maximum amount of hemocyanin that viscosity and colloid-osmotic effects will allow (up to 200 mg hemocyanin per ml of blood; Mangum, 1990), the carrying capacity of coleoid blood is less than half that of fish (Pörtner, 1994). Consequently, coleoids must maintain elevated cardiac outputs to meet the elevated oxygen requirements of their active tissues (Shadwick et al., 1990). Cephalopod hearts will not contract unless they are first filled (Hill and Welsh, 1966; Versen et al., 1997) and therefore venous return must be maintained to ensure adequate cardiac output. How do coleoid cephalopods ensure venous return?

Our understanding of coleoid cardiovascular function is incomplete. One outstanding issue is the relative importance of the mantle (the large muscular body wall that forces water over the gills), the hearts, and the contractile veins in driving venous return in intact coleoids. The mantle encloses the large veins and the hearts (Tompsett, 1939), much as the mammalian thorax encloses the equivalent organs. Mantle contractions influence intravenous pressures more noticeably than contractions of other organs such as the renal appendages, and have been credited with driving venous return within the mantle cavity (Johansen and Martin, 1962; Bourne, 1987). However, to move blood between vascular areas within the mantle cavity, mantle contractions must generate pressure differences between those vascular areas. The pressures created by the mantle, although large at times, are probably applied equally to all vascular areas within the mantle. Therefore they would not create the pressure differences required to generate venous flow. Besides the intravenous pulse caused by the mantle, a second, shorter, overlaid pulse also is usually measured in the large veins. It is not usually considered to be propulsive, and often is attributed to the contractions of organs bordering the veins such as the gills or renal appendages (Johansen and Martin, 1962; Bourne, 1982). Another possibility, however, is that the large veins themselves might contract *in vivo*, creating this second pulse and also contributing to venous return. Indeed, the large veins have been found to contract *in vitro* (Williams, 1909; Tompsett, 1939; Schipp, 1987a), in dissected octopods (Smith and Boyle, 1983), and in anaesthetized octopods whose mantles were turned inside out (Wells and Smith, 1987). Early studies on cephalopod cardiovascular systems concluded that the system was driven solely by serial peristalsis between organs [Bert (1867), Fredericq (1914) and Skramlik (1929), as cited in Johansen and Martin, 1962; Wells and Smith, 1987]. Understanding what generates the propulsive forces is important for our understanding of cardiovascular function and energetics in cephalopods.

Despite our incomplete understanding of cardiovascular function in coleoids, experiments tapered off in the early 1990s. This is probably partly because coleoids are difficult to study using existing technology. Recent experiments have usually measured intravascular pressure or blood flow by implanting cannulae in the vasculature of unrestrained, unanaesthetized octopods (e.g. Johansen and Martin, 1962; Wells, 1979; Wells and Wells, 1983; Wells et al., 1987). Squid and cuttlefish are less well studied. We know of only two studies investigating pressures and flow through squid vessels (Bourne, 1982, 1984), and no studies investigating pressure or flow in cuttlefish vessels. The three *in vivo* studies on cuttlefish circulation measure only systemic heart function (Mislin, 1966; Chichery and Chanelet, 1972a; Chichery, 1980), possibly because the large internal shells of cuttlefish and their other viscera impede access to many of the veins (Chichery and Chanelet, 1972b).

Imaging technologies promise to improve our understanding of coleoid cardiovascular dynamics. Ultrasound imaging was

used by Tateno (1993) to visualize octopus mantles. We used ultrasound imaging to view the cardiovascular organs of unanaesthetized, unrestrained cuttlefish (*Sepia officinalis* L.) in real time. Ultrasound can be applied in any imaging plane and is non-invasive. Consequently, we were able to view different combinations of organs repeatedly, without harming the cuttlefish.

Our study sought to determine which organs contributed to venous return in resting cuttlefish, in order to clarify how cuttlefish circulatory power requirements are met. First, we established that we could reliably identify the large veins in cuttlefish using ultrasound imaging. Then, we determined whether the veins appeared to contract actively or to be compressed by other organs. We examined what role the veins might play in driving venous return. Finally, we evaluated the mantle's role in propelling the blood of resting coleoid cephalopods.

## Materials and methods

### *General housing conditions*

We obtained juvenile cultured *Sepia officinalis* L. from the National Resource Center for Cephalopods, Galveston, Texas, USA. We housed the cuttlefish in fiberglass home tanks, 78 cm×62 cm with a water depth of 25.5 cm. Black, opaque, plastic puck board divided tanks in half lengthwise, allowing two cuttlefish to be kept in each tank while remaining visually isolated from each other. Each side was supplied with water from an open seawater system at 2 l $min^{-1}$ (Dalhousie Aquatron). Water temperature was 21°C from September 2002 to December 2002 and 15°C from January 2003 to April 2003. Unless otherwise noted, all reported observations were made on sexually mature cuttlefish, 15.5–18.5 cm in mantle length, and kept at 15°C. The temperature in the experimental tank and the home tank was the same. The lights were on a 12 h:12 h dark:light cycle. We fed the cuttlefish thawed shrimp, fish or squid daily, *ad libitum*.

### *Experimental set-up*

We monitored physiological parameters using an ultrasound machine and a 5 MHz convex array ultrasound transducer (Ultramark 4 plus, Advanced Laboratory Technologies, Bothell, Washington, USA). Ultrasound transducers emit high frequency sound and interpret the reflected sound to create two-dimensional, real-time images of the internal organs of animals. Our transducer created images at approximately 17 frames $s^{-1}$. We could resolve vessels that were a few millimeters in diameter, which limited us to imaging the large veins (the arteries were too small to see). The transducer was held under the experimental tank and moved to capture the organs of interest. Ultrasound images (sonograms) were recorded on Hi-8 movie tape.

To reduce disruption to the cuttlefish during experiments, the experimental tank was divided into an inner and an outer compartment (Fig. 2). During experiments, we placed a single cuttlefish in the cylindrical (27 cm diameter, 14.5 cm high) inner compartment, where water depth was approximately 8 cm. This inner compartment had rigid plastic walls and a flexible, thin, white, plastic membrane across the bottom, upon which the cuttlefish could settle. Water entered the inner compartment through perforated airline tubing that ran around its bottom edge. Water then flowed through approximately 120 small holes (6 mm in diameter) in the walls of the inner compartment to the outer compartment, from which water was drained by a siphon. Water remained well aerated in the inner tank when a cuttlefish was present (>90% $O_2$ saturation).

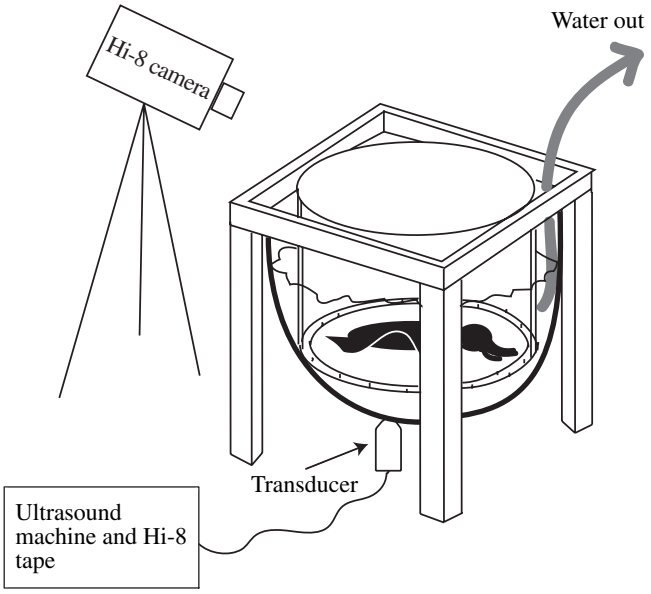


Fig. 2. Schematic diagram of the experimental set-up. Water was fed into the inner compartment of the experimental tank through perforated airline tubing. It then passed to the outer compartment through many small holes and was drained by a siphon. The cuttlefish (black) was placed in the inner compartment.

The outer compartment was made of flexible opaque plastic that hung from a rigid wooden frame, 29 cm×30 cm. To exclude sound-reflecting air from between the convex surface of the transducer and the surface of the outer compartment, consumer grade hand cream was applied to the transducer, which was then pushed into the soft plastic of the outer compartment. The water-filled space between the outer and inner compartments allowed the transducer to be operated without disturbing the cuttlefish.

The cuttlebone (the calcified skeletal structure found inside the dorsal body wall of the cuttlefish) is opaque to ultrasound. To avoid it, we insonated cuttlefish from below, through the acoustically transparent plastic bottoms of both the inner and outer compartments.

To visually isolate the cuttlefish from the rest of the room 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 the cuttlefish and to record its behaviour on Hi-8 movie tape during experiments. The camcorder movie

and sonograms were synchronized using audio cues recorded on both tapes.

### *Experimental protocol*

Each trial comprised seven 10 min physiological readings. The first reading started 30 min after the cuttlefish had been transferred into the experimental tank; before 30 min, cuttlefish moved too much to obtain the required sonograms. Subsequent readings started every 15 min, the last reading starting 2 h after the transfer. During each reading, we attempted to image one of the five organ groups described below for at least 20 s. Several trials were performed on the same cuttlefish, each separated by at least 2 days.

Circulatory organs are labeled in Fig. 1. Where possible, our nomenclature follows common usage (Williams, 1909; Tompsett, 1939; Hill and Welsh, 1966). We have added a region that we call the 'branch point' (BP). Although this point is probably not physiologically distinct from the lateral venae cavae, it clarifies subsequent discussion to note its location between the anterior vena cava and the arms of the lateral venae cavae. To measure contractions of the anterior vena cava, we identified two points along its length: Point A, near the opening of the mantle, and Point B, adjacent to the opening of the anus.

During each trial, we attempted to record images that contained the following organs simultaneously for at least 20 s (planes along which these sonograms were made are shown in Fig. 1): (1) A midsagittal section of the anterior vena cava and the mantle; (2) A midsagittal section through the branch point, the ventricle and the mantle; (3) A transverse section through the branch point, the efferent branchial vessels, the gills and the mantle; (4) A transverse section through the lateral venae cavae, the ventricle and the mantle; (5) A roughly transverse section through the ventricle, a branchial heart and the mantle.

In some trials, not all organ groups were imaged. Organ groups were not always imaged in the same order during a trial. Examples of sonograms can be viewed online as part of this article (see Movies 1 and 2 in supplementary material).

### *Data analysis*

Movie segments were eligible for analysis only if a stable image of one of the organ sets listed above was visible for at least 20 s. From these, we excluded movie segments in which cuttlefish were moving or showing non-resting body patterns (Hanlon and Messenger, 1996). For each day and each cuttlefish, we performed the analysis below on the single longest remaining movie segment for a given organ set. The entirety of this movie segment is referred to as an observation in the results.

We recorded the times $t$ (±1/15 s) of maximal contraction and maximal expansion for each organ of interest in the selected movie segment. Maximal contraction and expansion were determined by visually assessing the diameter of the vessel, heart or mantle. We calculated the phase shift between contractions of organs that were visible simultaneously and contracted at the same rate (differed by less than 4%) as follows:

$$\text{Phase shift} = (t_2 - t_1) / p_1 \times 360^\circ , \quad (1)$$

where $t_n$ is time of contraction of organ n and $p_n$ is period of organ n.

This calculation was performed for each contraction for a given organ pair of a given cuttlefish on a given day (i.e. for that observation). Using standard methods for circular statistics (Zar, 1999), we then calculated one average phase shift for each organ pair for that day and that cuttlefish (i.e. for that observation). The average phase shift measures the relative timing of the two organs' contractions; an average phase shift of approximately 0° or 360° indicates that the two organs tended to contract simultaneously, whereas an average phase shift of approximately 180° indicates a half-beat delay between the two contractions. To measure the consistency of average phase shifts, we used the circular statistic $r$ (Zar, 1999).

Let $\phi_1$ be the phase of contractions of organ 1, i.e.

$$\phi_1 = t_1 / p_1 \times 360^\circ , \quad (2)$$

and similarly for $\phi_2$. Now $r^2$ is the squared correlation for a linear regression (forced through zero) between the complex variables $z_1=e^{i\pi\phi_1/180}$ and $z_2=e^{i\pi\phi_2/180}$ [where $i=(-1)^{1/2}$], and resembles the coherence of conventional spectral analysis (Priestley, 1981). We calculated the statistical significance of average phase shifts from $r^2$ and the sample size using equation (1.3) of Greenwood and Durand (1955). Significance indicated that the phase shifts were more similar than we would expect by chance.

The Friedman test for trends (Zar, 1999) was used to test significance when order of contractions, rather than similarity of phase shift, was of interest.

If we could not see the contractions of organs that have been reported in the literature to contract, we measured the cross-sectional area of the organ using 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/) to ensure we were not overlooking contractions that were not large enough to be visible. For each cuttlefish, we made 21 measurements of the organ both when it was most likely to be contracted, and when it was most likely to be expanded. This was repeated three times for the same 21 contraction cycles. The mean difference between contracted and expanded organs was taken for each cycle. The standard deviation (S.D.) between the three replicates was taken for each cycle. Then we took the mean of the difference and of the S.D. over the 21 contraction cycles. The mean S.D. was taken as the measurement error. If the mean difference between expanded and contracted measurements was not greater than the measurement error, then we assumed there was no difference, i.e. that the organ did not contract.

### *Histology of vascular valves*

To find valves in the anterior and lateral venae cavae, we injected blue tracing medium into the vasculature of an

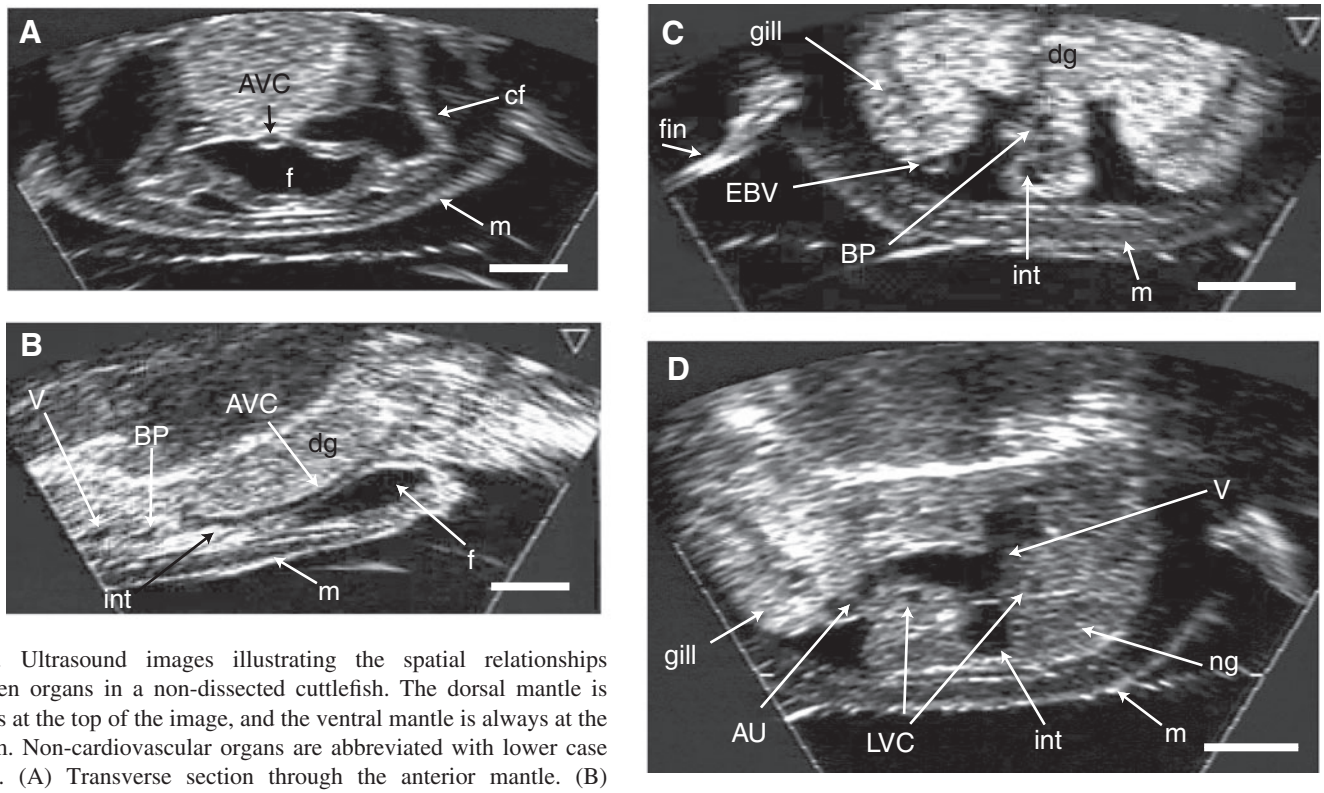


Fig. 3. Ultrasound images illustrating the spatial relationships between organs in a non-dissected cuttlefish. The dorsal mantle is always at the top of the image, and the ventral mantle is always at the bottom. Non-cardiovascular organs are abbreviated with lower case letters. (A) Transverse section through the anterior mantle. (B) Midsagittal section showing organs from planes 1 and 2 of Fig. 1 (see supplementary material, Movie 1). The head is towards the right. (C) Transverse section through the branch point and the efferent branchial vessels (plane 3, Fig. 1; also see supplementary material, Movie 2). (D) An oblique transverse section through the ventricle. Organs visible on the right are posterior to those on the left. This roughly corresponds to plane 4 in Fig. 1. Nidamental glands (ng) are present only in females. AU, auricle; AVC, anterior vena cava; BP, branch point; cf, collar flap; dg, digestive gland; EBV, efferent branchial vessel; f, funnel; int, shared course of the intestine and ink sac duct; LVC, lateral venae cavae; m, mantle; V, ventricle. All scale bars are 2 cm.

anaesthetized cuttlefish *via* the peribuccal sinus. Tracing medium (following Tompsett, 1939) was prepared by dissolving 60 g of melted gelatin and 6 g of potassium iodide in 60 ml of glycerol and 240 ml of 0.2% Alcian Blue (dissolved in 30% glacial acetic acid and 70% ethanol). Functionally, we identified a valve as a place where the tracing medium could be manually advanced along the vessel, but not pushed backward. We then dissected a 1 cm section of vasculature on either side of the putative valve and fixed it in neutral buffered formalin.

Next, we established the structure of the valve histologically. Tissue was dehydrated, cleared in CitriSolv (Fisher No. 22-143975, Nepean, ON, Canada), and embedded in low-melting paraffin wax. Embedded tissues were serially sectioned (5–7 μm) to obtain longitudinal sections, mounted on either Haupt's or poly-L-lysine coated slides, and stored at room temperature. Following rehydration, slides were stained with Masson's Trichrome (Flint et al., 1975). Stained slides were mounted in DPX (Fluka No. 44581, Oakville, ON, Canada), then viewed and digitally photographed on a compound microscope.

## Results

### *Representative sonogram images of the different organs*

Using ultrasound, we obtained the first movie images of cardiovascular organs moving in their relative positions in an intact, untethered cephalopod. The following images draw attention to the particular organs we identified. Organs appeared similar in all five cuttlefish, unless otherwise noted.

Organs were identified by their anatomical placement and connections. In the anterior part of the mantle cavity, the anterior vena cava was obvious in transverse views (Fig. 3A). In midsagittal views, it could be viewed in its entirety from the anterior end of the mantle to the branch point at the posterior end of the digestive gland (Fig. 3B; see Movie 1 in supplementary material). Anterior to the branch point, the anterior vena cava became small and indistinct in transverse views. The branch point was conspicuous in transverse view and its contractions were obvious (Fig. 3C and Movie 2 in supplementary material). It was harder to find in longitudinal section (Fig. 3B; see Movie 1 in supplementary material). The lateral venae cavae were distinguished by their thick walls (Fig. 3D). Their apparent wall thickness was likely due to their renal appendages. The ventricle appeared oblong and bent in transverse view (Fig. 3D). The shape and placement of the branchial hearts differed among cuttlefish. The efferent branchial vessels appeared as thin-walled structures on the distal edge of the gill (Fig. 3C).

Table 1. *The phase shift between the contractions of different organ pairs*

| | Mantle | | Point A (AVC) | | |
|---|---|---|---|---|---|
| Animal | Expansion – gill maximum | Contraction – gill minimum | Contraction – Point B (AVC) contraction | Expansion – mantle expansion | Contraction – mantle expansion |
| 11 | –62.4 ($N$=4, $r^2$=0.978, $P$<0.001) | –135.4 ($N$=4, $r^2$=0.761, $P$<0.05) | 88.3 ($N$=4, $r^2$=0.882, $P$<0.02) | –48.3 ($N$=4, $r^2$=0.902, $P$<0.01) | 129.3 ($N$=4, $r^2$=0.968, $P$<0.01) |
| 12 | –118.0 ($N$=3, $r^2$=0.996, $P$<0.01) | –149.1 ($N$=3, $r^2$=0.955, $P$<0.02) | 89.4 ($N$=4, $r^2$=0.949, $P$<0.01) | –153.4 ($N$=4, $r^2$=0.310, NS) | –1.3 ($N$=4, $r^2$=0.930, $P$<0.01) |
| 17 | –144.9 ($N$=4, $r^2$=0.982, $P$<0.001) | –161.6 ($N$=4, $r^2$=0.942, $P$<0.01) | 119.9 ($N$=4, $r^2$=0.980, $P$<0.001) | –96.0 ($N$=4, $r^2$=0.746, $P$<0.05) | 44.4 ($N$=4, $r^2$=0.944, $P$<0.01) |
| 22 | –139.6 ($N$=4, $r^2$=0.857, $P$<0.02) | –156.4 ($N$=4, $r^2$=0.766, $P$<0.05) | 59.6 ($N$=2, $r^2$=0.975) | –156.0 ($N$=2, $r^2$=0.917) | 31.2 ($N$=2, $r^2$=0.578) |
| 26 | Mantle movements not visible | Mantle movements not visible | 74.1 ($N$=4, $r^2$=0.898, $P$<0.01) | Mantle movements not visible | Mantle movements not visible |
| Average phase shift | –117.9 | –150.7 | 86.1 | –115.7 | 45.5 |
| $r^2$ between cuttlefish | 0.717 | 0.971 | 0.884 | 0.518 | 0.477 |
| $P$ | <0.05 | <0.01 | <0.01 | NS | NS |

Significance was only determined for sample sizes greater than 2.

*Variability of mantle and ventricle contraction rates*

Two hours after moving a cuttlefish to the experimental tank, we estimated the ventilation rate (mantle contractions) and heart rate (ventricular contractions) by counting the number of complete contractions in 10 s. At 21°C, the mean ventilation rate was 49.5±10.4 breaths min$^{-1}$, and the mean heart rate was 39.0±4.8 beats min$^{-1}$ ($N$=4 cuttlefish). The same four cuttlefish kept at 15°C had means of 33.0±6.6 breaths min$^{-1}$ and 22.5±1.9 beats min$^{-1}$, respectively, giving a $Q_{10}$ of 1.97 for ventilation rate and 2.50 for heart rate. However, this $Q_{10}$ must be interpreted with caution, because the cuttlefish were older and larger when kept at the lower temperature.

When the mantle and ventricle were observed simultaneously, their contraction rates were not well correlated at a given temperature (linear regression: 21°C: $r^2$=0.112; 15°C: $r^2$=0.005). At 15°C, the mantle usually contracted faster than the ventricle (30/37 observations, five cuttlefish). In 5/37 observations on five cuttlefish, the mantle and the ventricle shared the same contraction rate. Even when they had the same rate (5 observations, two cuttlefish), however, the phase shift between mantle and ventricle was not consistent within (4 observations, one cuttlefish, $r^2$=0.00015, NS) or between animals (two cuttlefish, $r^2$=0.1557, NS). Contractions of the heart and mantle have been found to be independent in previous studies on cuttlefish (Chichery and Chanelet, 1972a), octopus (Wells, 1979) and squid (Shadwick et al., 1990).

*Contractions of vessels and gills*

In all five cuttlefish kept at 15°C, we observed obvious contractions of the anterior vena cava, the branch point, the lateral venae cavae, the branchial hearts, the efferent branchial vessels, the auricles, the ventricle and the mantle. The veins were considered to be actively constricting (contracting) because they remained circular while their diameter decreased, rather than being deformed. Furthermore, the vessels were not being passively extended by a passing bolus of blood because there are no strongly pulsatile organs upstream of the veins that could create such a bolus of blood. Informal observations suggested that the auricles contracted approximately 180° out of phase with the ventricle.

The gills changed shape and moved back and forth within the mantle (supplementary material, Movie 2). To determine whether gill movements were synchronized with mantle movements, we recorded the times when the gills were farthest from the mid-line of the cuttlefish (‘maximum’) and when they were closest (‘minimum’). These were compared to the expansions and contractions of the mantle, respectively. The phase shift between the gill and mantle movements was consistent both within animals (4/4 cuttlefish where mantle movements could be accurately assessed, see Table 1) and between animals ($N$=4; maximum: $r^2$=0.7168, $P$<0.05; minimum: $r^2$=0.9708, $P$<0.01). Although the gills moved, we were unable to find evidence that they contracted. For each cuttlefish ($N$=5), we used the NIH Image program (version 1.62) to measure the transverse cross-sectional area of the gills at their ‘maxima’ and ‘minima’ for 21 consecutive oscillations. In 3/5 cuttlefish, the difference between the maximum and minimum areas was not greater than the measurement error (approx. 17 mm$^2$ out of approx. 550 mm$^2$). In the other two

cuttlefish, one showed a maximum that was slightly larger than its minimum (*G*-statistic for goodness of fit: mean difference=14.9 mm$^2$, $G$=11.9, d.f.=1, $P$<0.001) and in the other, the situation was reversed (*G*-statistic for goodness of fit: mean difference=–22.9 mm$^2$, $G$=15.9, d.f.=1, $P$<0.001). It seems unlikely that the gills contracted. If they did, gill contractions were not obvious and not synchronized with their movements within the mantle cavity.

Of the vessels observed, only the anterior vena cava contracted at the same rate as the mantle (18/18 observations, five cuttlefish). The ventricle always shared the contraction rate of the branch point (9/9 observations, five cuttlefish), the lateral venae cavae (19/19 observations, 5 cuttlefish) and the branchial hearts (13/13 observations, four cuttlefish; for a fifth cuttlefish, no data was obtained on the branchial hearts). We were unable to reliably capture an efferent branchial vessel and the ventricle in the same image. Therefore, the efferent branchial vessel's contraction rate was compared with that of the branch point, which always contracted at the same rate as the ventricle (see above). The efferent branchial vessel contracted in 21/26 observations (five cuttlefish). We might not have seen all instances of efferent branchial vessel contraction. We were more likely to see contractions when image quality was good, when we held the transducer at certain angles and when we looked at the end of the efferent branchial vessel that joined the auricle. Using the criteria established in the methods, we selected 17 of the instances that the efferent branchial vessel contracted in five cuttlefish for analysis. In 13 of these, the efferent branchial vessel had the same rate as the branch point. In the 4 observations in which the rates differed, results were split between the efferent branchial vessel contracting faster (4.4% and 4.6% faster), and the branch point contracting faster (8.4% and 19.7% faster). The efferent branchial vessel never contracted at the same rate as the mantle.

Therefore, there appear to be two groups of organs actively contracting at two different rates. In one group were the mantle and the anterior vena cava. In the other were the ventricle, the branch point, the lateral venae cavae, the branchial hearts and, usually, the efferent branchial vessels.

### *Relative timing of vascular contractions*

Vessels that contract at the same rate do not necessarily contract in an order that will propel blood. We investigated the timing of contractions along the anterior vena cava, and also between the branch point, lateral venae cavae and the branchial heart, to test whether contractions could be propulsive. Because these vessels and organs can only generate pressure gradients by contracting (they do not actively expand; Wells, 1978; Schipp, 1987a), only the relative times of contraction were considered.

Sonograms revealed that the anterior vena cava contracted in peristaltic waves that traveled posteriorly (see Movie 1 in supplementary material). The time of contraction of two points on the anterior vena cava (Fig. 1) were measured: Point A was close to the opening of the mantle, and Point B was at the opening of the anus. In 4/4 cuttlefish, there was a consistent

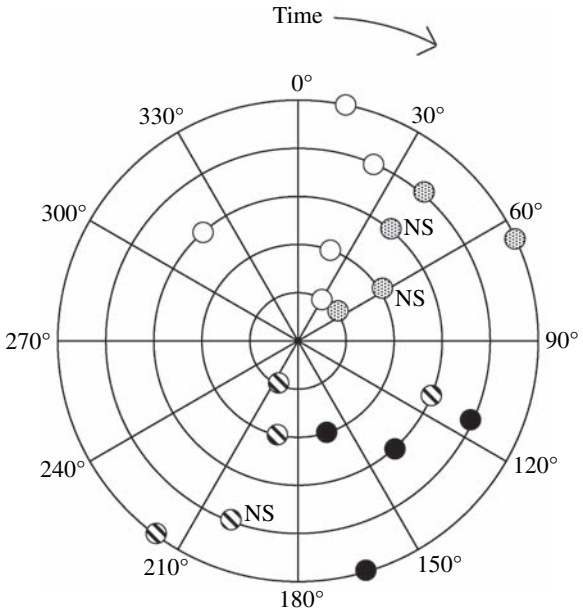


Fig. 4. Phase shift between the maximum contraction of the ventricle (arbitrarily set at 0°) and the branch point (open circles; BP in Fig. 1), the lateral venae cavae (grey circles; LVC in Fig. 1), the branchial hearts (black circles; BH in Fig. 1) and the efferent branchial vessels (hatched circles, EBV in Fig. 1). Time proceeds clockwise. Each concentric circumference shows the averaged data for one cuttlefish. NS indicates averages calculated from raw data that were not significantly similar (significance only determined if there were more than two data points. There were never more than four data points). Because data on the efferent branchial vessel and ventricle were not collected simultaneously, contraction phase of the efferent branchial vessel was calculated from its average phase shift from the average phase shift of the branch point. We obtained no data on the branchial heart for the cuttlefish of innermost circumference.

phase shift between the contractions of Points A and B both within (for a fifth cuttlefish, there were only two data points, so significance was not calculated; see Table 1) and among animals ($N$=5, $r^2$=0.88, $P$<0.01). Given that the period of the contractions of the anterior vena cava varied, it is perhaps not surprising that the speed of the peristaltic wave varied within and among cuttlefish. The lowest mean speed for a given cuttlefish was 0.05 m s$^{-1}$ (range: 0.04–0.06 m s$^{-1}$, 4 observations), and the highest was 0.1 m s$^{-1}$ (4 observations). Cuttlefish #26 had the highest variability in speeds of peristaltic contraction along the anterior vena cava (0.05–0.08 m s$^{-1}$, 4 observations). However, speed did not vary with the period of contraction (linear regression: 18 observations, 5 cuttlefish, $r^2$=0.47, $P$=0.197, NS).

To determine the relative contraction times of the branch point, the lateral venae cavae and the branchial heart, we first arbitrarily designated the maximal contraction of the ventricle as 0°. We had already determined the phase shift between contractions of the other three organs and the ventricle, and we used the ventricle as a reference point to reconstruct the order of contractions of the other organs. The average phase of the efferent branchial vessel was calculated from its phase shift

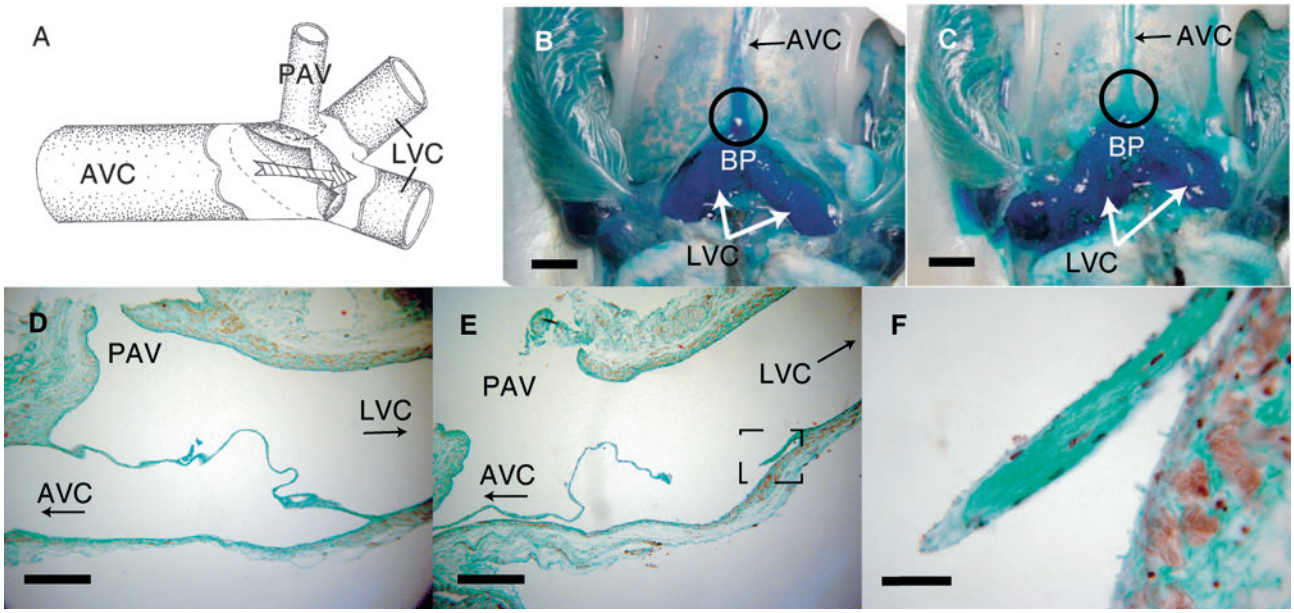


Fig. 5. (A) Schematic representation of the valve when open. Blood travels from the anterior vena cava (AVC) into the lateral venae cavae (LVC). When blood pressure rises in the LVC relative to the AVC, the valve closes. (B) Blue tracing medium in the AVC (circled). (C) Once tracing medium was pushed from the AVC (circled) into the branch point, it could not be pushed back into the AVC. Scale bar, 1 cm. (D) Close to the lateral wall, the valve spanned the whole vessel (×6 magnification; scale bar, 0.5 mm). (E) Mid-sagittally a natural split occurred in the valve tissue. We verified that it was not an artifact through analysis of successive serial sections. The distal end of the larger portion of the valve tissue was reinforced by a polysaccharide-rich thickening (×6 magnification; scale bar, 0.5 mm). (F) The muscular, small side of the valve, indicated by a broken box in E. Muscle cells stained red (×60 magnification; scale bar, 50 μm). AVC, anterior vena cava; BP, branch point; LVC, lateral venae cavae; PAV, posterior azygos vein.

from the branch point. The resulting phase shifts are shown in Fig. 4. When taking the smallest arc in which contractions of the branch point, lateral venae cavae and branchial heart occurred, the organs consistently contracted in the following order (Fig. 4): branch point, lateral venae cavae, branchial heart (Friedman test: $N$=4, $\chi^2_r$=8, $P$<0.05; for the fifth cuttlefish no information was obtained for the branchial heart). Apparently, a peristaltic wave travels from the branch point to the branchial hearts in live, intact cuttlefish.

The efferent branchial vessels usually contracted shortly after the branchial heart, and almost 180° before the ventricle (average phase, 3/4 cuttlefish, Fig. 4). The contractions of the efferent branchial vessels might aid with auricular or ventricular filling.

### *Anatomical separation between anterior vena cava and branch point*

Since the branch point and the anterior vena cava contract at different rates (BP and AVC of Fig. 1), the branch point will occasionally contract when the anterior vena cava is expanding. This might push blood anteriorly towards the head, instead of posteriorly towards the hearts. We found a previously unidentified valve in this location that prevented flow reversal (Fig. 5A).

Tracing medium could be pushed from the anterior vena cava into the branch point. When we applied pressure to the lateral venae cavae or branch point, however, the tracing medium did not flow back into the anterior vena cava (Fig. 5B,C).

The presence of a valve was confirmed in histological section. Successive serial longitudinal sections of the anterior and lateral venae cavae were examined to reconstruct the structure of the valve (Fig. 5A). The valve was composed of a large flap of thin cellular valve tissue with a shallow, off-center slit in it. The tissue was attached obliquely in the vessel along the dorsal and one of the lateral walls, and part way along the ventral wall. Where it attached to the vessel walls, the valve tissue was 2–3 cells thick, and was supported by extensive extracellular fibres. These fibres stained green with Masson's trichrome, indicating the presence of fibrous proteins, which could be collagen (Flint et al., 1975). The remainder of the tissue was usually only one cell thick (Fig. 5D). Where it was split, the edge of the larger portion of the valve was reinforced by polysaccharide-rich extracellular matrix (Fig. 5E). Polysaccharides were selectively stained blue by the acidic Alcian Blue dye used in the tracing medium (Klymkowsky and Hanken, 1991). Muscle cells were interspersed within the fibrous matrix in the smaller portion where the valve split (Fig. 5F). Muscle cells stained red. We did not stain specifically for nerves.

### *Role of mantle in circulation*

Previous researchers have suggested that the mantle might drive venous return, especially through the anterior vena cava

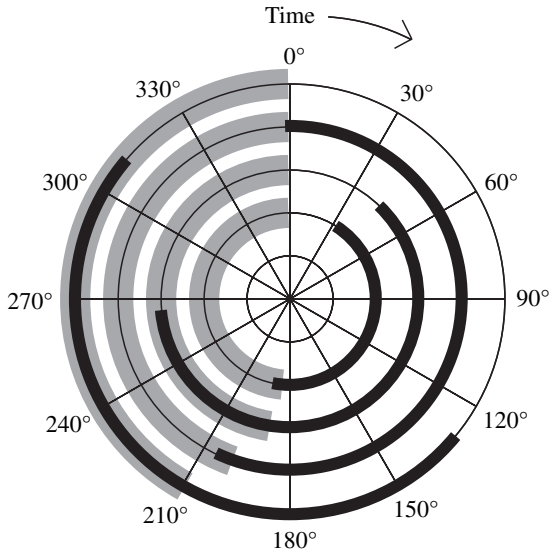


Fig. 6. Phase shifts between the contraction cycle of the mantle and the contraction cycle of Point A on the anterior vena cava (AVC). Full expansion of the mantle is arbitrarily set at 0° and time proceeds clockwise. The heavy black line starts at the full contraction of Point A (AVC) and ends at the full expansion of Point A. The heavy grey line starts at full mantle contraction and ends at full mantle expansion. Each concentric circumference represents the averaged data of a different cuttlefish.

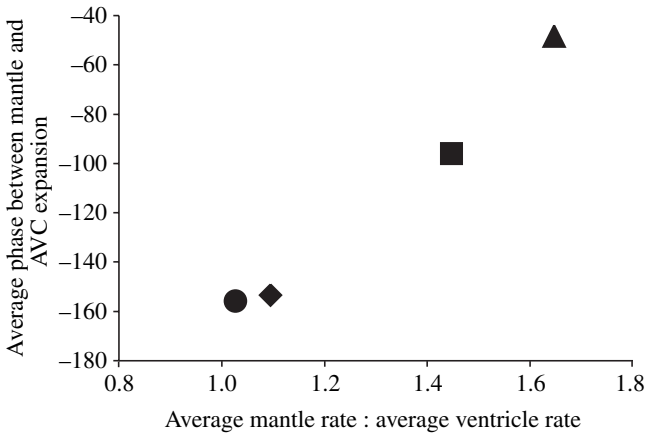


Fig. 7. The phase shift between the mantle and the anterior vena cava (AVC) plotted against the ventilation rate: heart rate ratio. Fig. 6 shows the variation in the phase shift between mantle and AVC contractions between animals. This variation is largely explained by how many times the mantle contracts per heart contraction (linear regression: $r^2$=0.98, $P$=0.0082, $N$=4). Each symbol represents averaged data from one cuttlefish.

(Johansen and Martin, 1962; Bourne, 1982, 1987). Indeed, the anterior vena cava was the only vessel that contracted at the same rate as the mantle in our study. Therefore we investigated the mantle's role in driving blood through the anterior vena cava.

The phase shift between the expansion of the mantle and the beating of Point A on the anterior vena cava was usually consistent within cuttlefish (Table 1, contraction: 3/3 cuttlefish; expansion: 2/3 cuttlefish; in a fourth cuttlefish, there were only two data points, so significance was not determined). In 3/4 cuttlefish, the contraction of the anterior vena cava (Point A) was almost 180° out of phase with the contractions of the mantle (Fig. 6). Therefore, the mantle is not simply compressing the anterior vena cava.

The phase shift between the anterior vena cava and the mantle, although consistent within cuttlefish, was not consistent among cuttlefish (Table 1: $N$=4; Point A expansion: $r^2$=0.5183, NS; Point B expansion: $r^2$=0.4767, NS). In other words, when the mantle was fully expanded, the anterior vena cava could be one quarter contracted in one cuttlefish, but entirely contracted in another (Fig. 6, two outermost circumferences). Therefore, none of the organs or vessels investigated were synchronized with the mantle in a way that was consistent between cuttlefish. Nevertheless, mantle dynamics may somehow interact with vascular function. Earlier, we noted that one group of organs contracted with the mantle's rate (anterior vena cava) and another group contracted with the ventricle's rate (lateral venae cavae, branchial hearts and efferent branchial vessel). The ratio of contraction rates between the mantle and the ventricle groups was highly correlated to the phase shift between mantle and anterior vena cava expansion (Fig. 7; $N$=4, $r^2$=0.9837, $P$=0.0082). The reason for this is unclear.

## Discussion

The large veins (the anterior vena cava, the lateral venae cavae and the efferent branchial vessels) have been observed to contract *in vitro* (Williams, 1909; Tompsett, 1939; Schipp, 1987a), in dissected octopods (Smith and Boyle, 1983), and in anaesthetized octopods whose mantles were turned inside out (Wells and Smith, 1987). Wells and Smith (1987) proposed that all veins, except the anterior vena cava, contract actively and contribute to venous return. However, venous function in intact coleoid cephalopods has been difficult to study using implanted pressure transducers (Chichery and Chanelet, 1972b; Bourne, 1982, 1987; O'Dor et al., 1990; Shadwick et al., 1990; Pörtner et al., 1991) and, until now, non-invasive techniques have not been applied to this system. Using ultrasound, we have made the first non-invasive measurements of cardiovascular function in unanaesthetized, free-moving, intact cephalopods, and the first measurements of any kind on vascular function in cuttlefish. All the large veins, including the anterior vena cava, contracted actively in resting cuttlefish.

As in octopods (Smith, 1962), the cuttlefish anterior vena cava pulsed at the same rate as the mantle. In our cuttlefish, the anterior end of the anterior vena cava usually contracted when the mantle was expanding, and *vice versa*. Therefore the anterior vena cava does not appear to be compressed by the mantle in resting cuttlefish. Furthermore, contractions of the anterior vena cava were peristaltic, traveling posteriorly towards the branch point. We conclude that the anterior vena

cava contracts actively and peristaltically to propel blood towards the branch point. The peristaltic waves of the anterior vena cava of *S. officinalis* traveled at speeds of 0.04–0.1 m $s^{-1}$, slightly slower on average than the 0.1 m $s^{-1}$ reported previously for the arm veins of the octopus *Enteroctopus dofleini* Wülker (Smith, 1962). The variable speed of peristalsis along the anterior vena cava suggests that its speed might be influenced by nervous or hormonal input. Variable speeds might help synchronize venous contraction with ventilatory activity.

The anterior vena cava contracts at a different rate than the branch point. Consequently, contractions of the branch point could occasionally push blood back towards the anterior vena cava. We discovered a valve that prevents backflow from the branch point to the anterior vena cava, thereby ensuring that blood flows only in the proper direction. We suggest this valve be called the Wells valve in recognition of his influential pioneering work on cephalopod circulation. Many vascular valves in crustaceans (Wilkens, 1997; Davidson et al., 1998) and octopods (Smith and Boyle, 1983) are both muscular and innervated. The Wells valve was muscular, but we do not know whether it was innervated. If innervated, it could regulate cardiac output by controlling blood flow into the lateral venae cavae and therefore into all three hearts; like other molluscan hearts, the systemic heart of coleoids adjusts its cardiac output according to venous return (Wells and Smith, 1987). Furthermore, by regulating flow into the lateral venae cavae, the Wells valve could regulate the pressure in the renal appendages, which are involved with solute exchange between the blood and the forming urine (Martin and Harrison, 1966).

Once past the Wells valve, blood was propelled by a second peristaltic wave. This wave started at the branch point, traveled along the lateral venae cavae, and ended with the contraction of the branchial hearts. Because there is a valve at both ends of the lateral venae cavae (the Wells valve and one at the entrance to the branchial heart), the lateral venae cavae could act as auricles to the branchial hearts.

The gills moved within the mantle, probably in response to mantle-driven water movement. However, unlike previous authors (Johansen and Martin, 1962; Bourne, 1982), we found no evidence that the gills contracted in intact cuttlefish. We saw no contractions and could not detect contractions when we measured the cross-sectional area of the gills at their closest and furthest points from the cuttlefish's midline. If the gills did contract, contractions were either less than 3% of the gill's transverse area (measurement error), or were not synchronized with their movements within the mantle. The gills might still contribute to circulation; preliminary evidence suggests that, *in vitro*, individual gill lamellae or vessels within the lamellae contract to propel blood through the branchial capillaries (Wells and Smith, 1987).

After passing through the gills, blood appeared to be forced into the auricles by contractions of the efferent branchial vessels. These contractions were especially evident in the section of efferent branchial vessel connected to the auricles. Contractions usually had the same frequency as ventricular contractions, but always a different frequency than mantle contractions. Unlike octopods (Johansen and Martin, 1962), the efferent branchial vessel is probably not an extension of the auricle in cuttlefish because there is a valve separating the efferent branchial vessel and auricle in cuttlefish (Versen et al., 1997). Cephalopods have maximized the numbers of contractile veins, likely to ensure ample venous return to feed the elevated cardiac output of the coleoid heart.

The lateral venae cavae, branchial heart, efferent branchial vessels and ventricle all contracted with the same frequency, and in a specific order. These contractions were not simply driven by contractions of the mantle, which had a different frequency. Furthermore, the lateral venae cavae and the branchial hearts are on one side of the branchial capillary bed, whereas the efferent branchial vessels and the ventricle are on the other side. Therefore, the rate and order of contractions was not driven by simple serial peristalsis between organs, as was suggested by early authors [Bert (1867), Fredericq (1914) and Skramlik (1929), as cited in Johansen and Martin, 1962; Wells and Smith, 1987]. The nervous system probably plays a role in coordinating contractions; an investigation into the innervation of this area revealed that the lateral venae cavae, the branchial heart, the efferent branchial vessels, the auricles and the ventricle are all connected by nerves in octopods (Smith, 1981; Smith and Boyle, 1983). The auricles may set the rate of ventricle (Versen et al., 1997). It has been suggested that an element in the ventricle is responsible for establishing the contraction rate of all these interconnected organs (Wells and Smith, 1987). Our results raise the possibility that such a region might instead be in the branch point (Fig. 4).

Both heart rate and ventilation rate decreased with decreasing temperature. Adapted $Q_{10}$ values for heart and ventilation rate were, respectively, 2.50 and 1.97. These values might be confounded by uncontrolled age and size effects, but they are similar to $Q_{10}$ values for heart rate and ventilation rate in *Octopus vulgaris* Cuvier (Wells, 1979) and the squid *Lolliguncula brevis* Blainville (Wells et al., 1988).

We have assumed that the mantle does not compress venous vessels directly in resting cuttlefish in part because none of the vessels' contractions were timed to the mantle's movements in a way that was consistent between cuttlefish. However, new evidence suggests that although mantle cavity pressure changes have the same frequency as mantle movements, they are not timed to the ventilatory period in a way that is consistent between cuttlefish (F. Melzner, personal communication). If this is true, the anterior vena cava, but none of the other veins observed in our experiments, may contract when the mantle pressure is high. Even if contractions of the anterior vena cava are timed to pressure increases within the mantle cavity, however, it does not mean that mantle pressure is directly compressing the anterior vena cava. The anterior vena cava contracts peristaltically towards the posterior of the cuttlefish, and the pressures created during resting ventilation likely travel anteriorly above the anterior vena cava. The fact remains that none of the veins contracted in a way that was consistent with the pressures produced by the mantle. Instead,

the coordination between the contractions of the anterior vena cava and the pressures in the mantle may facilitate venous return from the head and arms during the low venous and mantle pressures that coincide with anterior vena cava and mantle expansion (Smith, 1962; Wells et al., 1987).

It is not to say that mantle contractions have no possible role in circulation. Contractions of the mantle and anterior vena cava might be linked to contractions of the rest of the vasculature. The mantle and anterior vena cava contracted at the same rate, and all other investigated organs contracted at the same rate as the ventricle. The ratio of contractions between mantle and ventricle groups is strongly correlated with the phase shift between the onset of mantle contraction and the onset of anterior vena cava contraction. However, the nature of the connection between these organ groups is obscure.

The interaction between the heart and the mantle is modified during cephalopod jetting. During octopus jetting, the heart stops (Wells et al., 1987), and during squid jetting, heart rate increases (Shadwick et al., 1990). It is unclear how jetting will affect cuttlefish circulation. Further investigations into mantle vasculature dynamics, peripheral vascular resistance, vessel pliability and regional pressure changes within the mantle cavity (especially if they are synchronized with sonograms) might clarify the mantle's role in circulation in both resting and jetting cuttlefish.

Like vertebrate cardiovascular systems, coleoid cephalopod cardiovascular systems are proving to be complicated, certainly much more complicated than a series of vessels that propel blood simply by serial peristalsis, or a system driven solely by a systemic heart (Wells, 1978; Schipp, 1987b). In some regards, coleoid circulation is strikingly mammalian. This convergence has been shaped by similar factors (Packard, 1972; O'Dor and Webber, 1986); however, the original *Bauplan* of each group has resulted in important and interesting differences between these groups. Non-invasive technologies such as ultrasound provide us with tools to further the investigation of this sophisticated but poorly understood invertebrate circulatory system.

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) (S.A.A., A.G.C. and A.J.K.), a Dalhousie University Graduate Fellowship (A.G.C.) and the Lett fund (A.J.K.). We thank Greg Breed for his artwork in Fig. 5A and Laura Weir for digitizing movies for inclusion online. We thank Dr Ron O'Dor, Andrea Ottensmeyer and Frank Melzner for comments on the manuscript that have improved its content in many places.

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