COLEOID CEPHALOPOD STRATEGIES FOR POWERING VENOUS RETURN, RESPONDING TO SUDDEN VISUAL STIMULI AND REGULATING MALE AGONISTIC BEHAVIOUR

by

Alison J. King

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To

All those who have had a dream, and have had the courage to chase it

and

All those who helped those dreamers on their way

and to

My great-grandmother Mather who encouraged us all to academic pursuits.

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Abstract

All animals with complex behaviours and closed circulatory systems face similar biological challenges. Well-studied examples of solutions to these challenges come primarily from the vertebrates. However, coleoid cephalopods (Phylum Mollusca) also have complex behaviours and closed circulatory systems. They provide examples of solutions that, while functionally similar to the solutions of the vertebrates, are structurally different. I investigated the structure of coleoid strategies for powering venous return, responding to sudden stimuli and regulating male agonistic behaviour.

Studies into cardiovascular function in coleoids, and especially in cuttlefish, have been limited using traditional techniques. I used ultrasound to image the circulatory organs of the cuttlefish, *Sepia officinalis*, for the first time. All the large veins (anterior and lateral venae cavae and efferent branchial vessel) contracted in unanaesthetized, untethered, resting cuttlefish. The anterior vena cava and lateral venae cavae contracted peristaltically. I discovered a muscular valve which separated these two unsynchronized vessels and ensured blood flowed in only one direction between them. Contractions of the veins were not timed to mantle movements in a way that was consistent between cuttlefish. Therefore, venous return is likely to be powered by venous contraction in resting cuttlefish, and not by pressures produced inside the mantle cavity by ventilation.

Like other animals, coleoids need to respond to novel, potentially threatening, stimuli in their environment. When resting cuttlefish were presented with a sudden visual stimulus, ventilation and heart rates fell below resting levels. Drops in ventilation and heart rates were inversely proportional to resting rates. Cuttlefish also showed components of the Deimatic Display (including behavioural freezing) and hyperinflated their mantles during the response. The Deimatic Display is part of cuttlefish predator avoidance behaviour. Behavioural freezing (including decreased ventilation rate) may help cuttlefish to prepare for flight by increasing sensory acuity. Mantle hyperinflation helps to prepare cuttlefish for flight by filling the mantle with water prior to escape jetting. Decreased heart rate may be a product of the unusual arrangement of muscles and capillaries in the cuttlefish mantle.

Male squid actively compete for mates. Squid are group-living, and are therefore continuously surrounded by both conspecific males and females. Because male-male competition is costly, we expect squid to limit competition to times of highest potential reproductive benefit. I found that paired male squid (*Loligo pealeii*) increased agonistic behaviour in the presence of conspecific egg mops (a group of gelatinous egg capsules, containing eggs that are frequently already fertilized). Squid found the egg mops by sight, but agonistic behaviour only increased after squid touched the egg mop with their arms. Visual cues from the egg mop maintained elevated agonistic behaviour between touches. Agonistic behaviour subsided within 10 min of egg mop removal.

Cuttlefish and squid share several functional traits with the vertebrates; they have closed circulatory systems that are functionally similar to those of mammals and birds, responses to sudden stimuli that, like in other animals, include behavioural freezing and decreased ventilation and heart rate, and, in squid, ways to regulate male-male competition. However coleoids are distinct from vertebrates because they have many contractile veins, because decreased heart rate after a sudden stimulus in cuttlefish is likely due to the unique arrangement of capillaries and muscles in their mantle, and because squid use the unusual cue of fertilized eggs to regulate male-male competition.

Acknowledgements

Long gone are the days of Newton, when one (albeit very brilliant) person, working in isolation, can do good science. With the expansive and integrative nature of current-day Biology, I would not have been able to study cephalopods in this depth without the help and influence of many people.

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Chapter 1: Introduction

1.1. The convergent evolution of cephalopods and fish

Millions of years of 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). One striking convergence is that both reduced their protective but heavy outer armor, thereby reducing their cost of transportation (O'Dor and Webber, 1991). In coleoid cephalopods, this was accomplished by reducing and internalizing the ancestral molluscan shell. Adopting an internal shell has had important ramifications. The mantle (body wall) has become highly muscular and the mass of water ejected from the mantle cavity (space that opens to the outside and is between body wall and viscera) has risen from 15% of the body mass in the hypothetical ancestral cephalopod, to 25% in modern cuttlefish and 50% in some modern squid (Wells, 1994). Ejecting more water has increased the power and speed of coleoid swimming (O'Dor and Webber, 1991). It has also increased mantle oxygen demands. In modern coleoids and fish, we now find closed circulatory systems that deliver oxygen more efficiently to the tissues than the open circulatory systems of their ancestors (Packard, 1972).

No longer possessing a protective shell, coleoids have had to develop behavioural defenses against predation from piscine, reptilian, avian and mammalian predators (Packard, 1972), such as escape jetting (Hanlon and Messenger, 1996). Other aspects of coleoid behaviour have also become more complex. No longer enclosed under a shell, the skin of coleoids has become capable of rapid, neurally-controlled chromatic and

textural changes (Hanlon and Messenger, 1996). These changes can be used in many ways, including predator avoidance by camouflage and signaling between males during mating behaviour (Hanlon and Messenger, 1996).

The increased complexity of the modern coleoids' circulatory systems and behaviour has solved some of the coleoids' biological challenges (e.g. oxygen delivery, predator avoidance, social communication), but has resulted in challenges of its own. Coleoids face the same sorts of challenges that face all animals with active life-styles, closed circulatory systems and complex behaviours. Some of the biological solutions to similar challenges function similarly between vertebrates and coleoids. For example, coleoids have developed eyes with focusing lenses and dilating pupils, similar to vertebrate eyes. They also have a sensory system on their arms that detects water currents, and is analogous to the lateral line system of fish (Hanlon and Messenger, 1996). Indeed, the many similarities between the solutions of coleoids and fishes prompted Packard (1972) to declare that "cephalopods functionally are fish". However, the coleoid solutions to their evolutionary challenges are shaped by their molluscan heritage (Packard, 1972; O'Dor and Webber, 1986), and are strikingly different from their vertebrate counterparts. In this thesis, I investigate the details of the coleoid strategies for powering venous return, responding to sudden stimuli and regulating male agonistic behaviour.

The second and third chapters of the thesis focus on coleoid circulation. In order to orient the reader to important background information, section 1.2. explains the anatomy and innervation of the coleoid circulatory system, its perceived limitations, and the limits to our current knowledge about it. Sections 1.3. to 1.5. introduce the topics of the thesis in more detail.

1.2. Cephalopod circulation

1.2.1. Similarities with vertebrates

Modern coleoids have circulatory systems that are uniquely efficient among the generally open circulatory systems of the molluscs (Fig. 1.1). They have two well-developed, single-chambered branchial hearts, one at the base of the each gill, small venous hemocoels (Packard, 1972), and true capillaries in the tissues (Williams, 1909; Tompsett, 1939; Bourne et al., 1978; Schipp, 1987a). Some endothelial-less sinuses remain in coleoids (Schipp, 1987a); however, coleoids are nonetheless described as having closed circulatory systems (Williams, 1909; Wells, 1978, Fig. 1.1.B.). The appearance of the branchial hearts, which pump blood through the gills, in addition to the systemic heart which pumps blood through the body (Tompsett, 1939), makes the coleoid circulatory system functionally similar to those of birds and mammals (Packard, 1972, Fig. 1.1.B. vs. D). The schematics in figure 1.1. illustrate the structural and functional differences between general molluscan and coleoid circulatory systems, as well as how coleoid circulatory systems compare to piscine and mammalian or avian systems.

1.2.2. Coleoid circulatory anatomy and control

This section describes the general structure of the coleoid circulatory system and the general structure of its control systems. The circulatory systems of octopus, squid and cuttlefish are similar; anatomical differences are noted where relevant. Circulatory organs are labeled in Fig. 1.2. Where possible, my nomenclature follows common usage (Williams, 1909; Tompsett, 1939; Smith, 1962; Hill and Welsh, 1966). I have added a region that I call the "branch point" (BP). Although this point is probably not

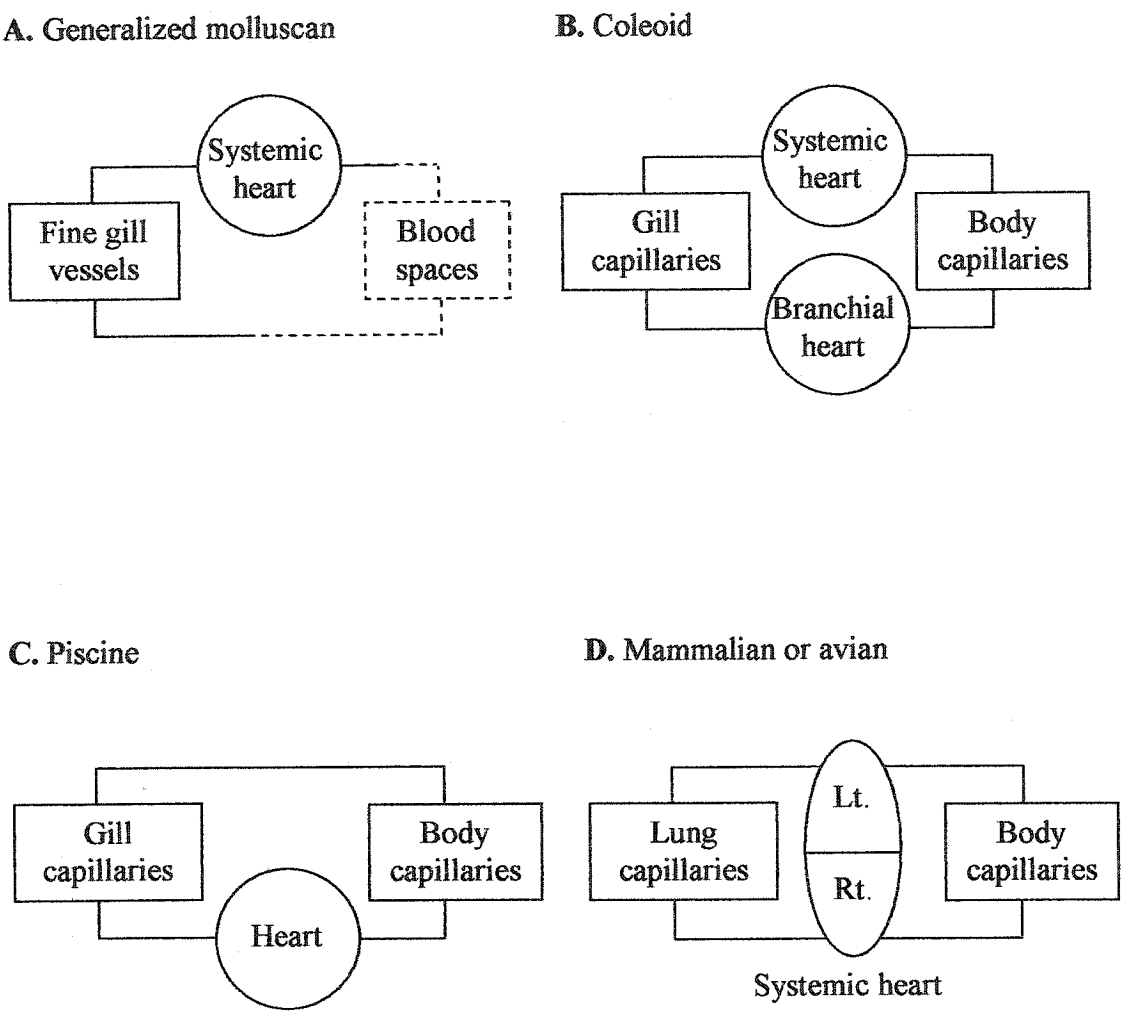


Figure 1.1. Schematic representations of molluscan and vertebrate circulatory systems. A. Generalized molluscan circulation. B. Coleoid circulation. C. Piscine circulation. D. Mammalian or avian circulation. Dashed lines represent open blood spaces; solid lines represent closed blood spaces. Blood flows clockwise through the structures.

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. The anterior vena cava and lateral venae cavae should be considered physiologically distinct (Wells and Smith, 1987).

Blood follows two major circuits through the coleoid body: the systemic circulation and the branchial circulation. The systemic circulation is powered by the systemic heart. The systemic heart consists of two thin-walled auricles and a muscular ventricle, which are separated by paired semilunar valves. Blood leaves the ventricle through two large aortae, and several smaller aortae. The two large aortae, the cephalic aorta and the posterior aorta, are each guarded by a semilunar valve. The aortae give off numerous arteries which in turn divide to form arterioles and finally capillaries. In squid, so-called "peripheral hearts" exist at the exits of large arteries from the mantle. Despite their name, peripheral hearts are not hearts but muscular valves that probably contract to occlude their arteries, preventing blood from rushing to the head and fins during mantle contraction (Williams, 1909). There is no mention of similar organs in *Sepia* or in *Octopus*. After passing through the capillaries, blood is collected in venules, small veins and venous sinuses, and is funneled towards the large veins. The best studied of the large veins is the anterior vena cava (Fig. 1.2.: AVC), which drains blood from the anterior regions of the animal and runs across the mantle cavity on the surface of the digestive gland. Many veins feed the anterior vena cava; their openings, as well as the anterior end of the vena cava at the margin of the mantle, are guarded by valves. The anterior vena cava bifurcates at the branch point to form the paired lateral venae cavae (Fig. 1.2.: LVC), which are covered with renal outpocketings.

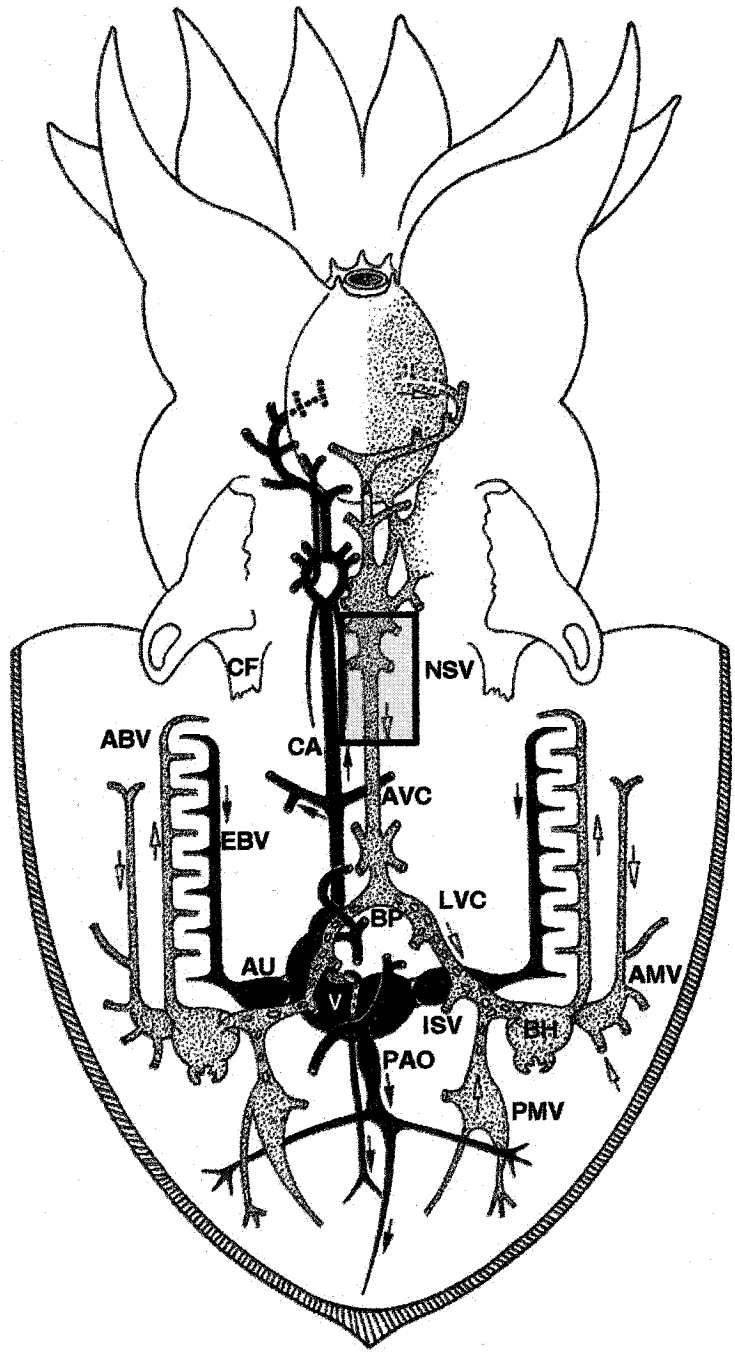


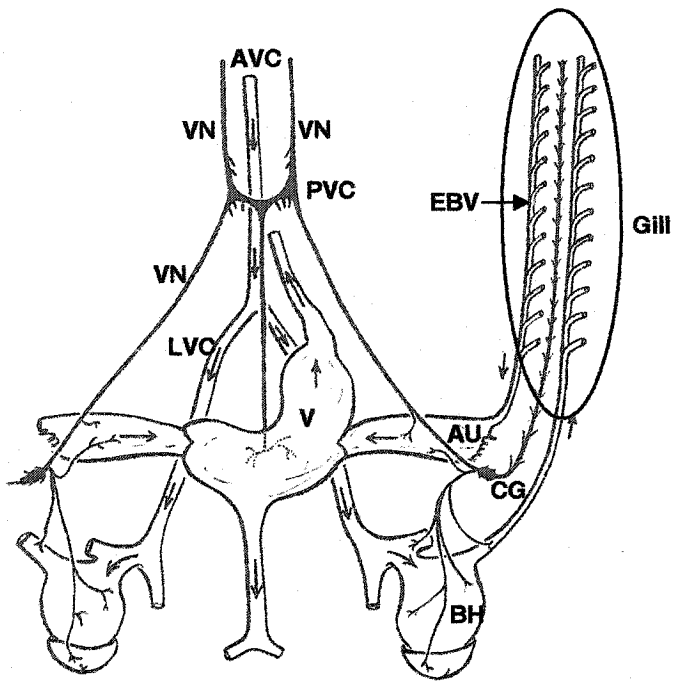
Figure 1.2. Schematic diagram of the circulatory system of *Sepia* (after Schipp, 1987b). ABV, afferent branchial vessel; AMV, anterior mantle vein; AU, auricle; AVC, anterior vena cava; BH, branchial heart; BP, branch point; CA, cephalic aorta; CF, collar flap; EBV, efferent branchial vessel; ISV, ink sac vein; LVC, lateral venae cavae; PMV, posterior mantle vein; POA, posterior aorta; V, ventricle. Green shaded region, area occupied by the neurosecretory system of the vena cava (NSV; after Alexandrowicz, 1965). Stippled vessels carry deoxygenated blood; dark vessels carry oxygenated blood.

Both on their way to the branchial hearts, and at the opening of the branchial heart, the lateral venae cavae receive the other large veins, namely the anterior and posterior mantle veins (Fig. 1.2.: AMV and PMV), the inksac vein (Fig. 1.2.: ISV) and the left mesenteric vein.

The second circulatory circuit, the branchial circulation, starts with the contractile branchial hearts (Fig. 1.2.: BH). The entrance of these is guarded by a pair of semilunar valves that prevent backflow into the large veins (Smith and Boyle, 1983). The branchial hearts pump blood into the afferent branchial vessels (Fig. 1.2.: ABV). In decapods, backflow from the afferent vessels into the hearts is prevented by fleshy valves (cuttlefish, Tompsett, 1939) or tubercles (squid, Williams, 1909). There is no valve in this location in octopods (Johansen and Martin, 1962; Houlihan et al., 1982). The afferent vessels branch and feed the capillaries of the gills, which are ultimately drained by the efferent branchial vessels (Fig. 1.2.: EBV). These feed directly into the auricles of the systemic heart.

The hearts and the peripheral circulatory system are well innervated. Neurons destined for the circulatory system stem primarily from the visceral lobe of the subesophageal part of the brain and travel in the paired visceral nerves (Tompsett, 1939; Wells, 1978; Schipp, 1987b), although vasomotor neurons arise from other places as well (Alexandrowicz, 1964; Smith and Boyle, 1983). Traveling parallel to the anterior vena cava, the paired visceral nerves extend towards the hearts (Fig. 1.3.). The pattern of branching and coalescing of the nerve fibers around the branchial and systemic hearts is complex, and will not be recounted in detail here (but see Smith & Boyle, 1983). It is enough to note that the lateral venae cavae, branchial hearts, gills, efferent branchial

A.



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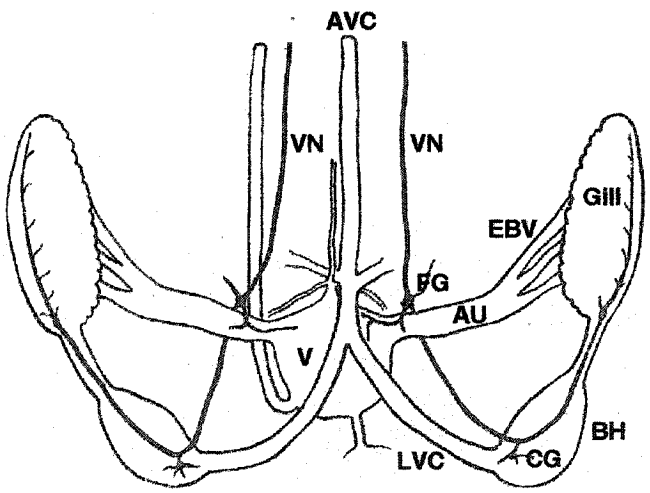


Figure 1.3. Schematic diagrams of the innervation of coleoid circulatory systems (after Hill and Welsh, 1966). A. Decapod innervation, dorsal view. B. Octopus innervation, ventral view.
AU, auricle; AVC, anterior vena cava; BH, branchial heart; CG, cardiac ganglion; EBV, efferent branchial vessel; FG, fusiform ganglion; LVC, lateral venae cavae; PVC, posterior visceral commissure; V, ventricle; VN, visceral nerve.

vessels and ventricle are interconnected by nerves and receive input from the visceral nerve (Smith and Boyle, 1983).

In addition to this extensive innervation, coleoids possess a neurosecretory tissue that releases cardio-active substances into the venous blood of the anterior vena cava. It is analogous to the neurohypophysis of vertebrates and is called the neurosecretory system of the vena cava (Martin and Voigt, 1987, Fig. 1.2.: NSV). See Alexandrowicz (1964; 1965) for reviews of its structure.

This extensive central control, using both neurosecretion and direct innervation, probably serves two functions. Many parts of the circulatory system contract in isolation, although some, most notably the auricles and ventricle, need to be filled before they contract (Wells, 1978). The input from the CNS probably allows some degree of central control over these independently contractile parts of the circulatory system. At the same time, connections between organs probably allow sensory feedback between hearts, gills, excretory and reproductive organs, allowing some degree of local control and coordination (Wells, 1978).

1.2.3. The challenges of jet propulsion and oxygen delivery in coleoids

Several authors have argued that the coleoid circulatory system is inferior to the vertebrate design (Wells and Smith, 1987; Mangum, 1990; O'Dor and Webber, 1991), largely because of the low oxygen carrying capacity of the blood. The low carrying capacity of the blood is exacerbated by the high oxygen demands of coleoid tissues. Coleoids use energetically expensive jet propulsion (Alexander, 1977) to generate thrust during swimming. Forward thrust is proportional to the mass multiplied by the velocity

of the water pushed backward (Wells, 1994). Because fish have wide sweeping tails, they can develop thrust by imparting low velocity to a large mass of water. In contrast, the limited aperture of the cephalopods' funnel requires them to impart a high velocity to a small mass of water (O'Dor and Webber, 1991). Because the cost of imparting velocity increases with the square of the velocity produced (O'Dor and Webber, 1991; Wells, 1994), cephalopods must expend more energy, and therefore consume more oxygen, than fish to achieve the same thrust (O'Dor and Webber, 1991). Some cephalopods, such as *Sepia* and *Loligo*, have reduced their costs of routine swimming by using fins to create thrust, like fish, instead of using typically cephalopod jet propulsion. Even so, the oxygen demand of routine swimming with fins is still higher in coleoids than it is in salmon, partially because coleoid fins are not as efficient as fish fins (O'Dor and Webber, 1991).

One of the tasks of coleoid circulation is to meet the oxygen demands of their active tissues. Circulatory oxygen delivery in coleoids is limited because their blood has a low oxygen carrying capacity. Cephalopods use hemocyanin as an oxygen transport protein. Hemocyanin is always extracellular, and not packaged in cells like vertebrate hemoglobin. The maximum concentration of hemocyanin in the blood is limited by increases in blood viscosity and colloid-osmotic pressure (Mangum, 1990). Even at maximum hemocyanin concentrations, the oxygen carrying capacity of the blood is only between 3.8 and 4.5 vol % (Redfield and Goodkind, 1929; Wells, 1978; Houlihan et al., 1982). In fish, these values are at least twice as high, usually around 6-15 vol % (Hill and Wyse, 1988); in mammals, this value is usually above 8 vol % and can reach almost 20 vol % (Withers, 1992).

In order to meet their oxygen demands, coleoids remove 80-90% of the modest amount of oxygen from their blood during each circuit, even when at rest (Wells, 1994).

This puts coleoids on the edge of oxygen peril; there is very little additional oxygen they could extract from the blood should circulation ever stop or should oxygen demands increase, as they do during jetting (Withers, 1992). To meet increased oxygen demands during jetting, coleoids are forced to greatly increase their cardiac output. During exercise, heart rate can double (squid) and stroke volume can increase by 50% (*Octopus*) to 300% (squid, Wells et al., 1987; Shadwick et al., 1990). Increases in heart rate and stroke volume, combined with increasing arterial pressures (Wells and Smith, 1987), result in the work and power output values of coleoid cardiac tissue rivaling or exceeding those of mammals (Shadwick et al., 1990; O'Dor and Webber, 1991). It seems likely that oxygen distribution mechanisms other than the systemic heart are at work during jetting; however, this hypothesis has been difficult to assess. By understanding mechanisms that aid oxygen distribution in resting coleoids, we may better understand which mechanisms could also function during exercise.

In coleoids, organs other than the hearts might help to circulate blood. There is evidence that contractions of the veins themselves or of peripheral organs, such as the gills and renal appendages, might help to propel blood (Schipp, 1987a). On a larger scale, the ventilatory contractions of the powerful mantle, which encloses most of the cardiovascular system, could influence hemodynamics by the pressures it creates in its mantle cavity or its tissues (Johansen and Martin, 1962; Shadwick et al., 1990). Unfortunately, the circulatory roles of the veins, the peripheral organs and the mantle have not yet been determined.

1.2.4. Limits to current knowledge about cardiovascular function in coleoid groups

In a ground-breaking study, Johansen & Martin (1962) implanted catheters in several blood vessels of unrestrained, conscious *Enteroctopus dofleini*, to investigate blood pressure. Their technique was a great improvement over earlier experiments, which involved nailing an animal to a board and slicing open its mantle (as described by Wells, 1978), and they inspired many papers about blood flow and its regulation in coleoids (e.g.: Johansen and Martin, 1962; Wells, 1980; Bourne, 1982; Wells et al., 1987). Despite our understanding of cardiovascular function in coleoids being incomplete, few experiments have been performed since the early 1990's. This is probably in part due to the difficulty of studying cephalopods, and especially squid (Bourne, 1987), using existing technology.

In octopods, the internal shell is essentially absent, giving easy access to the cardiovascular organs which lie directly against the internal shell in other coleoids. Octopods' cardiovascular organs can even be everted from the mantle and directly observed during surgery (Wells and Smith, 1987). Some species will tolerate several simultaneously implanted catheters well, although others use their prehensile arms to rip them out (Wells, 1978). Octopods also survive well in captivity. Because of the advantages of the octopus preparation, the information that exists on cardiovascular function in coleoids comes primarily from octopods (for review see Wells & Smith, 1987), and is the primary comparison for chapters 2 and 3.

Squids are less well-studied. They are constant swimmers that do not tolerate aquaria well (Bourne, 1987; Wells et al., 1988; Shadwick et al., 1990). Invasive studies

are few and limited in scope because many cardiovascular organs are inaccessible, being sandwiched between the reduced internal shell (the pen) and internal organs; because anaesthetics often result in death or poor recovery in squids; and because it is difficult to cannulate more than one vessel at a time (Bourne, 1982; Bourne, 1984; O'Dor et al., 1990; Shadwick et al., 1990; Pörtner et al., 1991). The cardiac performance of squid has been studied non-invasively by videotaping the systemic heart through the translucent dorsal mantle and pen (Wells et al., 1988; Shadwick et al., 1990). Used in conjunction with respirometers, this technique has yielded information on the work done by squid hearts during exercise. Unfortunately, it can only be used to evaluate the contribution of the systemic ventricle to circulation, and not the contributions of the veins or the mantle.

Cuttlefish are the least studied of the coleoids, probably because they have the largest internal shell, the cuttlebone. Flat and ovoid, it occupies most of the dorsal mantle creating a visually, sonically (personal observation) and electronically (Chichery and Chanelet, 1972b) opaque barrier dorsally, while viscera impede ventral access to all large cardiovascular structures, save the anterior vena cava (Chichery and Chanelet, 1972a). Only three studies exist on cardiac function in intact cuttlefish (Mislin, 1966; Chichery and Chanelet, 1972b; Chichery, 1980). All of these used invasive techniques that were limited to monitoring systemic heart function. Nothing is known of cuttlefish vascular dynamics.

1.3. Investigation of venous return in cuttlefish at rest

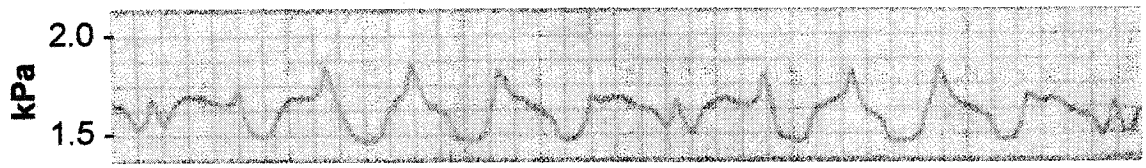
One of the important remaining questions about basic cephalopod circulation is how venous return is powered. Many authors working with implanted pressure transducers in both octopus (Johansen and Martin, 1962) and squid (Bourne, 1982) found that the large

veins (i.e. the anterior vena cava, the lateral venae cavae, and the efferent branchial vessels) experience two overlaid pressure pulses (e.g. Fig. 1.4.). The larger and presumably more important pulses corresponded with contractions of the mantle. They concluded that pressures produced in the mantle cavity during mantle contraction compressed the veins, driving blood to the hearts. They also concluded that, in most cases, that the smaller pulse was due to contractions of organs adjacent to the veins and that these pulses were not propulsive.

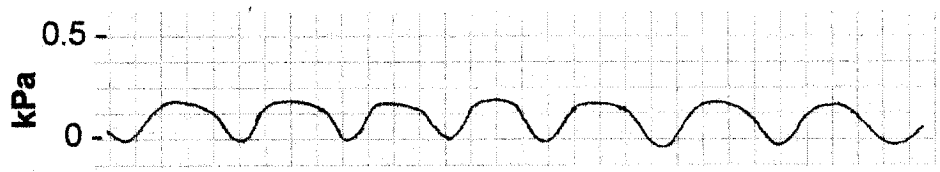
This is at odds with the findings of many recent anatomists, who find that the large veins contract autonomously *in vitro* (Williams, 1909; Tompsett, 1939; Schipp, 1987a), in dissected octopods (Smith and Boyle, 1983) and in anaesthetised octopods with their mantles turned inside out (Wells and Smith, 1987). The smaller pressure pulses measured by previous authors could have originated with the veins themselves, and could be at least partially responsible for venous return, given that many of the contractions are peristaltic. In their 1987 review, Wells and Martin hypothesize that all the large veins contract *in vivo*, except for the anterior vena cava, which they claim is the only vein to be compressed by the mantle. No one has been able to visualize the veins to investigate whether this is true.

Non-invasive imaging technologies promise to improve our understanding of coleoid circulation. Ultrasound has been used to image octopus mantles (Tateno, 1993), and could be used to study the large veins of resting cuttlefish for the first time. Ultrasound offers advantages over previous techniques in that we can observe whether the intravascular pressures recorded by previous authors result in vein deformation that is

A. Pressure trace from the efferent branchial vessel *in vivo*



B. Slow large pulse



C. Fast small pulse

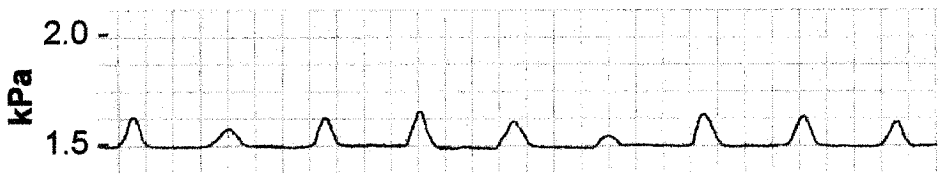


Figure 1.4. A. Pressure trace from the efferent branchial vessel measured by Johansen and Martin (1962). It consists of two superimposed pressure pulses of different frequencies: a slow and large pulse caused by ventilatory movements (B), and a faster and smaller pulse that they attribute to gill contractions (C) (see section 1.3 and 5.2.1. for discussion).

consistent with venous flow. In chapter 2, I used ultrasound to visualize the contractions of the large veins and the mantle. By comparing the timing of cardiovascular contractions to those of the mantle and the gills, I assessed the role these organs play in venous return in resting cuttlefish.

1.4. Cardiovascular and ventilatory responses to sudden stimuli

A strong evolutionary pressure during coleoid evolution was the need to evade reptilian and mammalian predators (Packard, 1972). When thinking about predator avoidance, most people envision the fight or flight response, during which cardiac output and ventilation rate are increased (Wingfield, 2003). However, sudden stimuli, including some that can signal the approach of a potential predator, result in decreased ventilation rate and decreased heart rate in animals of many different phyla (see Table 3.1.). This alternate response is also seen in octopods (Wells, 1980; Wells et al., 1987) and cuttlefish (Chichery, 1980). It is surprising that animals seemingly on the edge of oxygen peril would decrease oxygen uptake at the gills and its distribution through the circulating blood for any reason. In chapter 3, I investigated the ventilatory, cardiac, chromatic, postural and locomotory reactions of cuttlefish to sudden stimuli. By looking simultaneously at many different aspects of this response, I hoped to better understand the function of decreased ventilation rate and heart rate in the cuttlefish.

1.5. Regulation of agonistic behaviour

Male-male agonistic behaviour is costly (Enquist and Leimar, 1990; Lima and Dill, 1990; Marler and Moore, 1991; Isvaran and Jhala, 2000). Many group-living males

have cues that limit agonistic behaviour to times when it will be of the greatest potential reproductive benefit (Davies, 1991). Male squid live in groups and will fight for access to fertile females (Hanlon, 1996; DiMarco and Hanlon, 1997). Therefore, they are likely to regulate their agonistic behaviour. Squid have well-developed eyes, tactile chemoreception, distance chemoreception and a sensitive lateral-line analogue system (Hanlon and Messenger, 1996), and could use any combination of these to detect when their reproductive potential is highest. In chapter 4, I investigated cues that regulate male squid agonistic behaviour.

1.6. Synthesis of chapters 2 and 3

As discussed in section 1.2.3., the mantle produces large pressures in the mantle cavity during jetting. The mantle encloses the coleoid hearts, and many of the main blood vessels. Several have suggested that the pressures created in the mantle cavity affect circulation, especially during exercise (Johansen and Martin, 1962; Bourne, 1982). Additionally, the water moved through the mantle to produce thrust during jetting is used for respiratory gas exchange across the gills. Because of this, ventilation and locomotion are also linked. In other words, movements of the mantle not only produce the forces for locomotion, they also move water for ventilation and the resulting pressures may affect circulation. Chapters 2 and 3 present data on ways that the mantle and the circulatory system interact. In the discussion, I offer a new hypothesis about the connections between mantle movements and circulation in light of these new data.

1.7. Publications resulting from the thesis

Chapter 2:

King, A.J., Henderson, S.M., Schmidt, M.H., Cole, A.G. and Adamo, S.A. (in press). Using ultrasound to understand vascular and mantle contributions to venous return in the cephalopod *Sepia officinalis* Linnaeus. *J. exp. Biol.*

Chapter 4:

King, A. J., Adamo, S. A. and Hanlon, R. T. (2003). Squid egg mops provide sensory cues for increased agonistic behaviour between male squid. *Animal Behaviour* **66**, 49-58.

King, A. J., Adamo, S. A. and Hanlon, R.T. (1999). Contact with Squid Egg Capsules Increases Agonistic Behavior in Male Squid (*Loligo pealei*). *Biol. Bull.* **197**, 256.

Chapter 2: Using ultrasound to understand vascular and mantle contributions to venous return in the cephalopod *Sepia officinalis* Linnaeus

2.1. Linking information for this chapter

This chapter has been accepted by the *Journal of Experimental Biology*, pending changes suggested by the reviewers. The changes have also been incorporated into this chapter. The manuscript was co-authored with Stephen Henderson, Matthias Schmidt, Alison Cole and Shelley Adamo. Histological protocol and experiments were designed and executed by Alison Cole. She and I collaborated to interpret the data and write the relevant sections of the manuscript. For the ultrasound sections of this chapter, I designed the experimental set-up and protocol, performed the experiments and analysis, and wrote the manuscript.

2.2. 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 cuttlefish's blood to their 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* Linnaeus). 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 indirect and unclear connection with cardiovascular function. We conclude that, when cuttlefish are at rest, the mantle does not compress any of the large veins 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.

2.3. Introduction

Millions of years of exposure to similar selective pressures has 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 through the gills, powered by the single-chambered branchial hearts at the base of the each gill, and one through the body, powered by the ventricle and its two auricles (Fig.

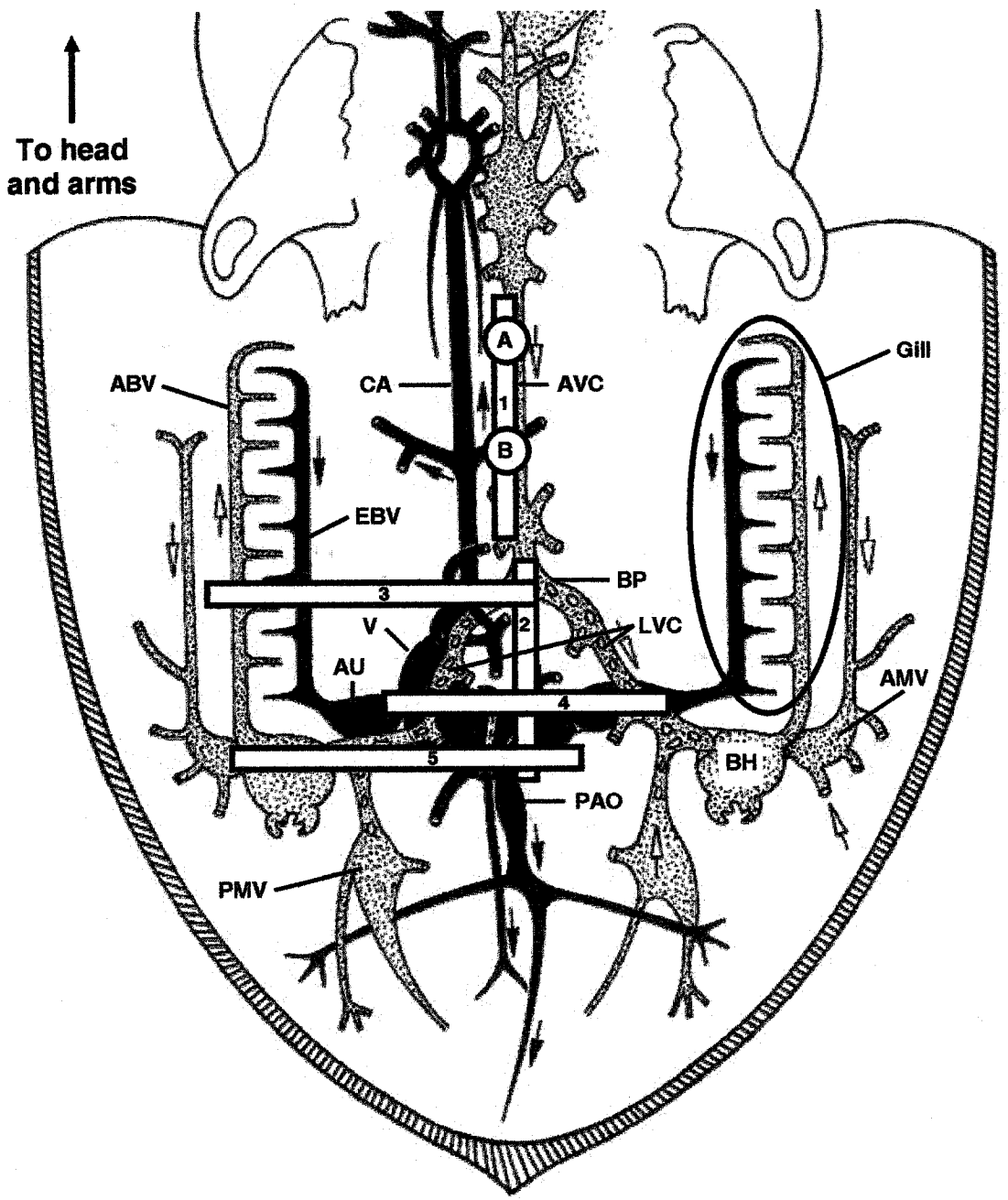
2.1.). In this regard, the coleoid circulatory system appears more avian or even mammalian than fish-like (Packard, 1972).

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, the 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, 1990; Pörtner, 1994). Despite having the maximum amount of hemocyanin that viscosity and colloid-osmotic pressures 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 the active coleoid 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?

Figure 2.1. The circulatory system of S. officinalis viewed from below (modified from Schipp, 1987). White rectangles indicate planes along which sonograms were taken. See 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; ISV, ink sac vein; LVC, lateral venae cavae; PMV, posterior mantle vein; PAO, posterior aorta; V, ventricle.

(Figure is on next page)


To head
and arms
A
B
ABV
CA
AVC
1
EBV
Gill
BP
3
V
2
LVC
AU
4
AMV
5
BH
PAO
PMV

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 bordering organs such as the gills or renal appendages (Johansen and Martin, 1962; Bourne, 1982). However, another possibility 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 anesthetized 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; Skramlik, 1929; as cited in Johansen & 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 1990's. This is probably in part 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 to (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 pressure and flow in squid vessels (Bourne, 1982; Bourne, 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, 1972b; Chichery, 1980), possibly because the large internal shells of cuttlefish and other viscera impede access to many of the veins (Chichery and Chanelet, 1972a).

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 conscious, unrestrained cuttlefish (*Sepia officinalis* Linnaeus) in real time. Ultrasound is non-invasive and can be applied in any imaging plane. 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 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.

2.4. Methods

2.4.1. General housing conditions

We obtained juvenile cultured *S. officinalis* Linnaeus from the National Resource Center for Cephalopods, Galveston, Texas, USA. We housed the cuttlefish in fiberglass home tanks, 78 x 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 sea-water system at 2 L/min (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 to 18.5 cm in mantle length, kept at 15°C. The temperature of the water in the experimental tank was the same as in the home tank. The lights were on a 12-hour dark, 12-hour light cycle. We fed the cuttlefish thawed fish or squid daily, *ad libitum*.

2.4.2. 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 per

second. We could resolve vessels that were a few millimeters in diameter. This 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 video tape.

To reduce disruption to the cuttlefish during experiments, the experimental tank was divided into an inner and outer compartment (Fig. 2.2.). During experiments, we placed a single cuttlefish in the cylindrical (27-cm wide, 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).

The outer compartment was made of flexible opaque plastic that hung from a rigid wooden frame, 29 x 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.

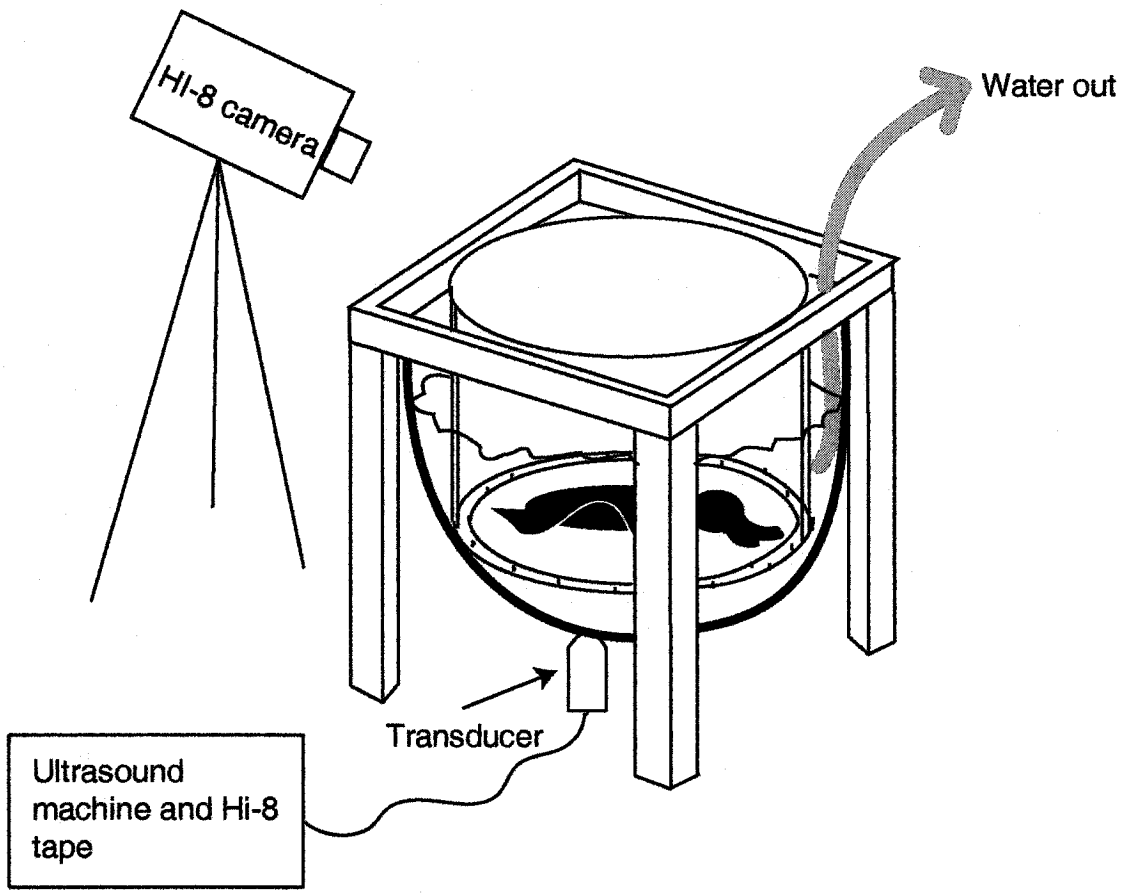


Figure 2.2. Schematic diagram of the experimental set-up for ultrasound trials. Water was fed into the inner compartment of the experimental tank through perforated airline tubing. It then passed to the outer compartment through many small holes and was drained by a siphon. The cuttlefish (black figure) was placed in the inner compartment.

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) 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 video tape during experiments. The camcorder video and sonograms were synchronized using audio cues recorded on both tapes.

2.4.3. Experimental protocol

Each trial comprised seven 10-minute 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 minutes, 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 20s. Several trials were performed on the same cuttlefish, each separated by at least two days.

Circulatory organs are labeled in Fig. 2.1. 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 seconds (planes along which these sonograms were made are shown in Fig. 2.1.):

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 planes were imaged. Organ groups were not imaged in the same order during a trial. Examples of sonograms are available on the CD accompanying this thesis (Videos 2.1. and 2.2.).

2.4.4. Data analysis

Video 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 video 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 video segment for a given organ set. The entirety of this video segment is referred to as an observation in the results.

We recorded the times (±1/15 s) of maximal contraction and maximal expansion for each organ of interest in the selected video segment. Maximal contraction and expansion were determined by visually assessing 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:

Equation 1:

$$\text{Phase shift} = \frac{\text{(time of contraction of organ 2 - time of contraction of organ1)}}{\text{Period of organ 1}} \times 360^{\circ}$$

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 cuttlefish. 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 ϕ_1 be the phase of contractions of organ 1, i.e.

Equation 2:

$$\phi_1 = \frac{\text{(time of contraction of organ 1)}}{\text{Period of organ 1}} \times 360^{\circ},$$

and similarly for ϕ_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 U.S. 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 average difference and standard deviation was taken for each measurement. Then we took the average of the averaged differences and of the standard deviations. The average standard deviation was taken as the measurement error. If the average 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.

2.4.5. Histology of vascular valves

To find valves in the anterior and lateral venae cavae, we injected blue tracing medium into the vasculature of a terminally anaesthetized cuttlefish via the peri-buccal 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% EtOH). 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), 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), coverslipped, then viewed and digitally photographed on a compound microscope.

2.5. Results

2.5.1. Representative sonogram images of the different organs

Using ultrasound, we obtained the first video 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. 2.3.A). 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. 2.3.B and Video 2.1.). 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

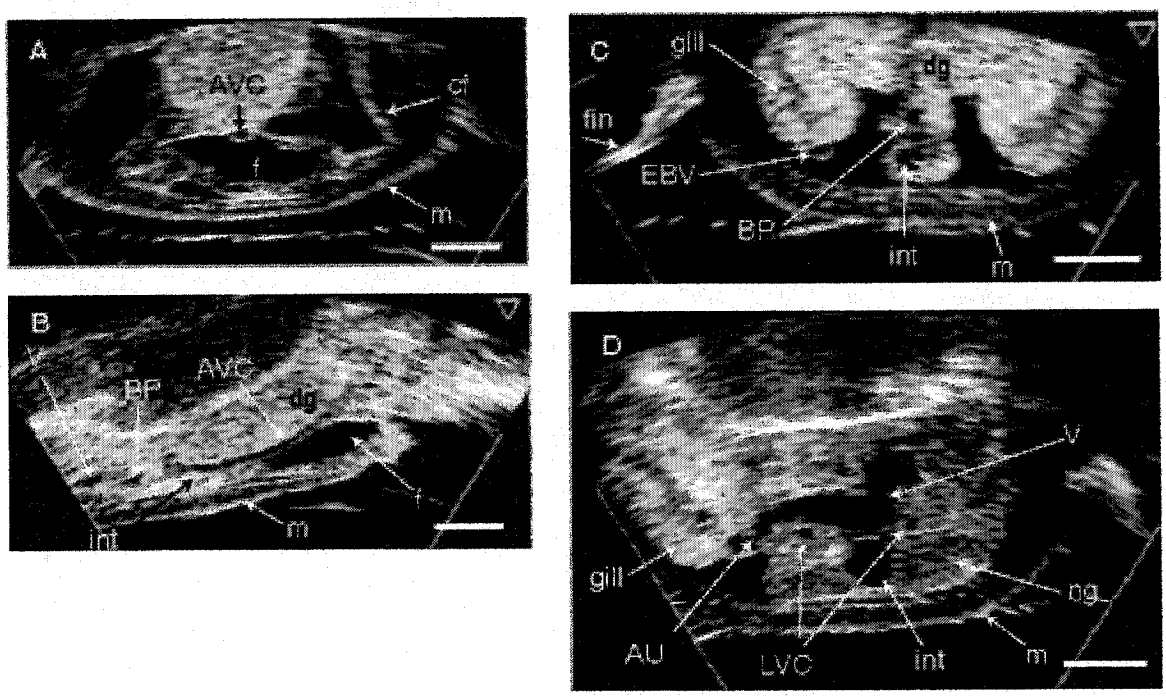


Figure 2.3. Sonograms illustrating the spatial relationships between organs in a non-dissected cuttlefish. The dorsal mantle is always at the top of the image, and the ventral mantle is always at the bottom. Non-cardiovascular organs are abbreviated with lower case letters. A. Transverse section through the anterior mantle. B. Midsagittal section showing organs from planes 1 and 2 of Fig. 2.1. (see Video 2.1.). The head is towards the right. C. Transverse section through the branch point and the efferent branchial vessels (plane 3, Fig. 2.1; also see Video 2.2.). 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, Fig. 2.1. All scale bars are 2 cm. Nidamental glands 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; ng, nidamental gland; V, ventricle.

its contractions were obvious (Fig. 2.3.C and Video 2.2.). It was harder to find in longitudinal section (Fig. 2.3.B and Video 2.1.). The lateral venae cavae were distinguished by their thick walls (Fig. 2.3.D). Their apparent wall thickness was likely due to their renal appendages. The ventricle appeared oblong and bent in transverse view (Fig. 2.3.D). 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. 2.3.C).

2.5.2. 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 average ventilation rate was 49.5 ± 10.4 breaths/min, and the average heart rate was 39.0 ± 4.8 beats/min (N=4 cuttlefish). The same four cuttlefish kept at 15°C had averages of 33.0 ± 6.6 breaths/min and 22.5 ± 1.9 beats/min 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 (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, 5 cuttlefish). In 5/37 observations on 5 cuttlefish, the mantle and the ventricle shared the same contraction rate. However, even when they had the same rate (5 observations, 2 cuttlefish), the phase shift between mantle and ventricle was not consistent within (4 observations, 1 cuttlefish, r^2=0.00015, NS) or between animals (2 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, 1972b), octopus (Wells, 1979) and squid (Shadwick et al., 1990).

2.5.3. 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. Informal observations suggested that the auricles contracted approximately 180° out of phase with the ventricle. The veins were considered to be actively constricting because they remained circular while their diameter decreased. Furthermore, the vessels are 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.

The gills changed shape and moved back and forth within the mantle (Video 2.2.). To determine whether gill movements were synchronised 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 2.1.) and among animals (N=4; maximum: r^2=0.7168, P<0.05; minimum: r^2=0.9708, P<0.01). Although the gills moved,

Table 2.1. The phase shifts between the contractions of different organ pairs. Significance was only determined for sample sizes greater than 2.

Animal	Mantle expansion– gill maximum	Mantle contraction – gill minimum	Point A (AVC) – Point B (AVC) (contraction)	Point A (AVC) expansion – Mantle expansion	Point A (AVC) 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=0.9684, 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)	-1.25 (N=4, r^2=0.9299, 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)	44.41 (N=4, r^2=0.9440, 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)	31.16 (N=2, r^2=0.5779)
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	-150.6509	86.08	-115.66	45.48
r^2 between cuttlefish	0.7168	0.9708	0.8842	0.5183	0.4767
P	<0.05	<0.01	<0.01	NS	NS

we were unable to find evidence that they contracted. For each cuttlefish (N=5), we used the NIH Image program (version1.62) to measure the transverse cross-sectional area of the gills at their "maxima" and "minima" as described above for 21 consecutive oscillations. In 3/5 cuttlefish, the difference between the maximum and minimum areas was not greater than the measurement error (appox. 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: average difference=14.9 mm^2, G=11.9, df=1, $P<0.001$) and in the other, the situation was reversed (G-statistic for goodness of fit: average difference=-22.9 mm^2, G=15.9, df=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, 5 cuttlefish). The ventricle always shared the contraction rate of the branch point (9/9 observations, 5 cuttlefish), the lateral venae cavae (19/19 observations, 5 cuttlefish) and the branchial hearts (13/13 observations, 4 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 brachial 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 (5 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. We analysed 17 of the instances that the efferent branchial vessel contracted in 5 cuttlefish.

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.

2.5.4. 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 (Video 2.1.). The time of contraction of two points on the anterior vena cava (Fig. 2.1.) 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 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 2.1.) 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 between cuttlefish. The lowest average speed for a given cuttlefish was 0.05 m/s (range: 0.04-0.06m/s, 4 observations), and the highest was 0.1 m/s (4 observations). Cuttlefish #26 had the highest variability in speeds of peristaltic contraction along the anterior vena cava (0.05-0.08 m/s, 4 observations). However, speed did not always vary with the period of contraction (Linear regression, 18 observations, 5 animals, 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 this to reconstruct the order of contractions. The average contraction time of the efferent branchial vessel was calculated from its phase shift from the average contraction time of the branch point. The resulting phase shifts are shown in Fig. 2.4. 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. 2.4.): branch point, lateral venae cavae, branchial heart (Friedman test: N=4, χ^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. 2.4.). The contractions of the efferent branchial vessels might aid with auricular or ventricular filling.

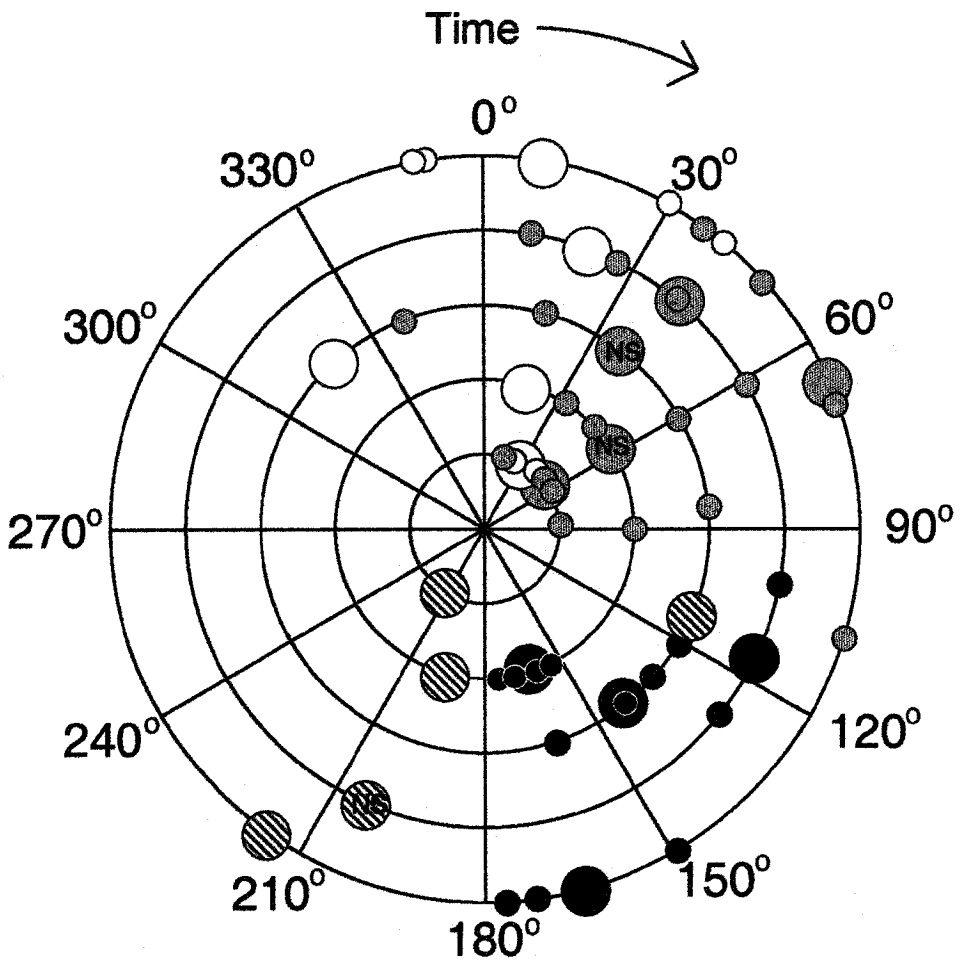


Figure 2.4. Phase shift between the maximum contraction of the ventricle (arbitrarily set at 0°) and the branch point (○; Fig. 2.1.: BP), the lateral venae cavae (◍; Fig. 2.1.: LVC), the branchial hearts (●; Fig. 2.1.: BH) and the efferent branchial vessel (▨; Fig. 2.1.: EBV). Time proceeds clockwise. Each concentric circumference shows the averaged data (large symbols) and raw data (small symbols) for one cuttlefish. NS indicates averages that were not significantly concentrated (significance only determined if there were more than two data points). Because data on the efferent branchial vessel and ventricle were not determined simultaneously, its contraction time was determined by its average phase shift from the average phase shift of the branch point. Consequently no raw data are shown. We obtained no data on the branchial heart for the cuttlefish of innermost circumference.

2.5.5. 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. 2.1.), 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 which prevented flow reversal (Fig. 2.5.A).

Tracing medium could be pushed from the anterior vena cava into the branch point. However, when we applied pressure to the lateral venae cavae or branch point, the tracing medium did not flow back into the anterior vena cava (Fig. 2.5.B, 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. 2.5.A). The valve was composed of 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. 2.5.D). Where it was split, the edge of the larger portion of the valve was reinforced by polysaccharide-rich extracellular matrix (Fig. 2.5.E). Polysaccharides were selectively stained blue by the acidic Alcian Blue dye used in the tracing medium (Klymkowsky and

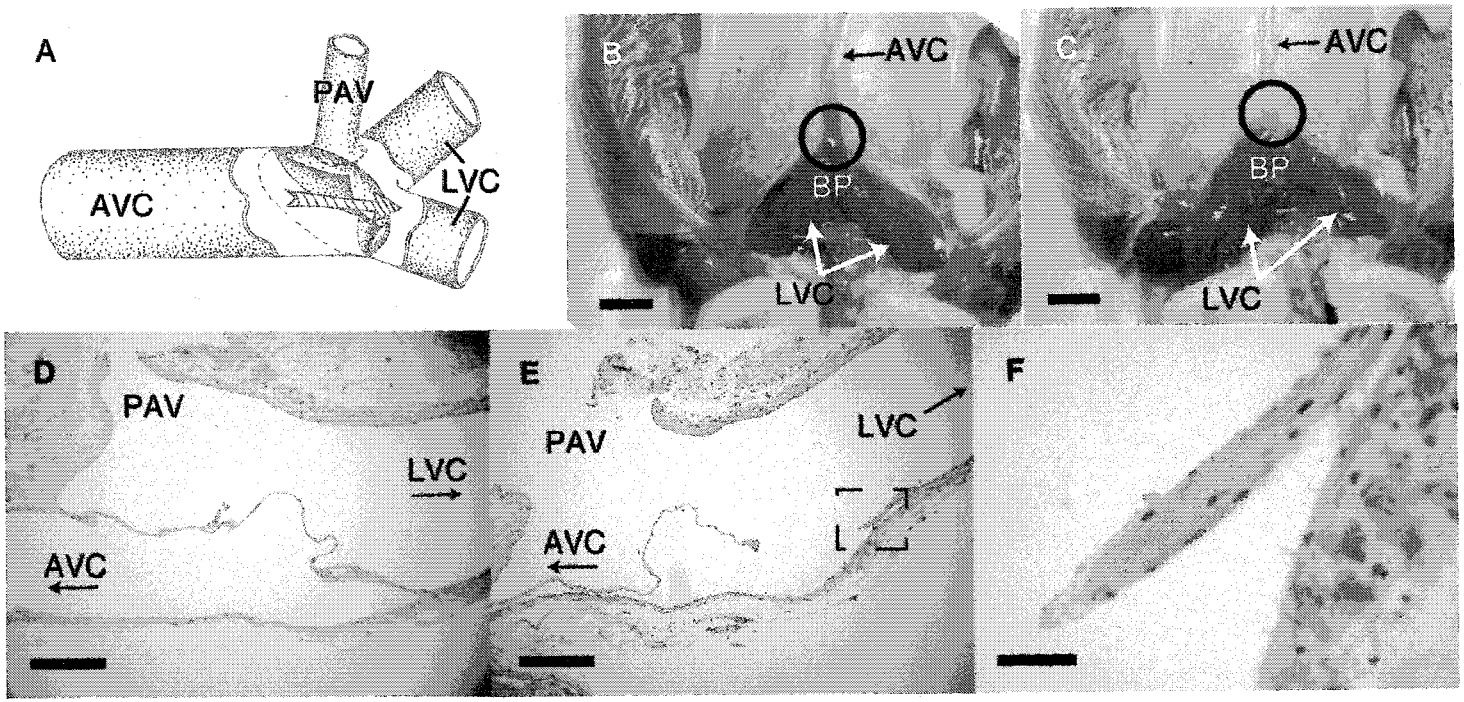


Figure 2.5. A. Schematic representation of the valve when open. Blood travels from the anterior vena cava (AVC) into the lateral venae cavae (LVC). When blood pressure rises in the LVC relative to the AVC, the valve closes. B. Blue tracing medium in the AVC (circled). C. Once tracing medium was pushed 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 (x6 magnification, scale bar: 0.5 mm). E. Midsagittaly a natural split occurred in the valve tissue. We verified that it was not an artifact through analysis of successive serial sections. The larger portion of the valve tissue was reinforced by a polysaccharide-rich thickening (x6 magnification, scale bar: 0.5 mm). F. The muscular, small side of the valve indicated by a dashed box in E. Muscle cells stained red (x60 magnification, scale bar: 50 μm).

AVC, anterior vena cava; BP, branch point; LVC, lateral venae cavae; PAV, posterior azygos vein.

Hanken, 1991). Muscle cells were interspersed within the fibrous matrix in the smaller portion where the valve split (Fig. 2.5.F). Muscle cells stained red. We did not stain specifically for nerves.

2.5.6. Role of mantle in circulation

Previous researchers have suggested that the mantle might drive venous return, especially through the anterior vena cava (Johansen and Martin, 1962; Bourne, 1982; Bourne, 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 2.1, 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. 2.6.). 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 2.1: 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 contacted in one cuttlefish, but entirely contracted in another (Fig. 2.6, 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

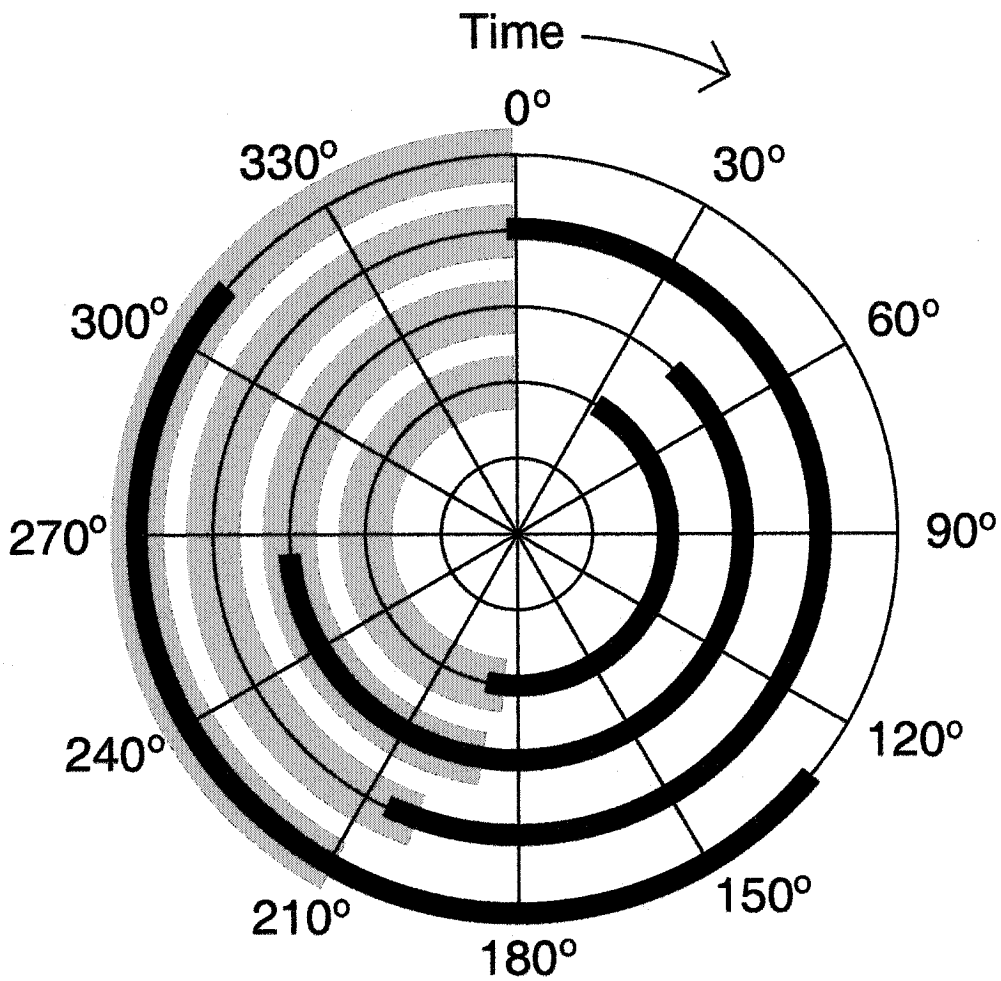


Figure 2.6. Phase shifts between the contraction cycle of the mantle and the contraction cycle of Point A on the anterior vena cava (AVC). Full expansion of the mantle is arbitrarily set at 0° and time proceeds clockwise. The heavy black line starts at the full contraction of Point A (AVC) and ends at 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.

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 group was highly correlated to the phase shift between mantle and anterior vena cava expansion (Fig. 2.7; N=4, r^2=0.9837, P=0.0082). The reason for this is unclear.

2.6. 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 anesthetized 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, 1972a; Bourne, 1982; Bourne, 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 undisturbed cuttlefish.

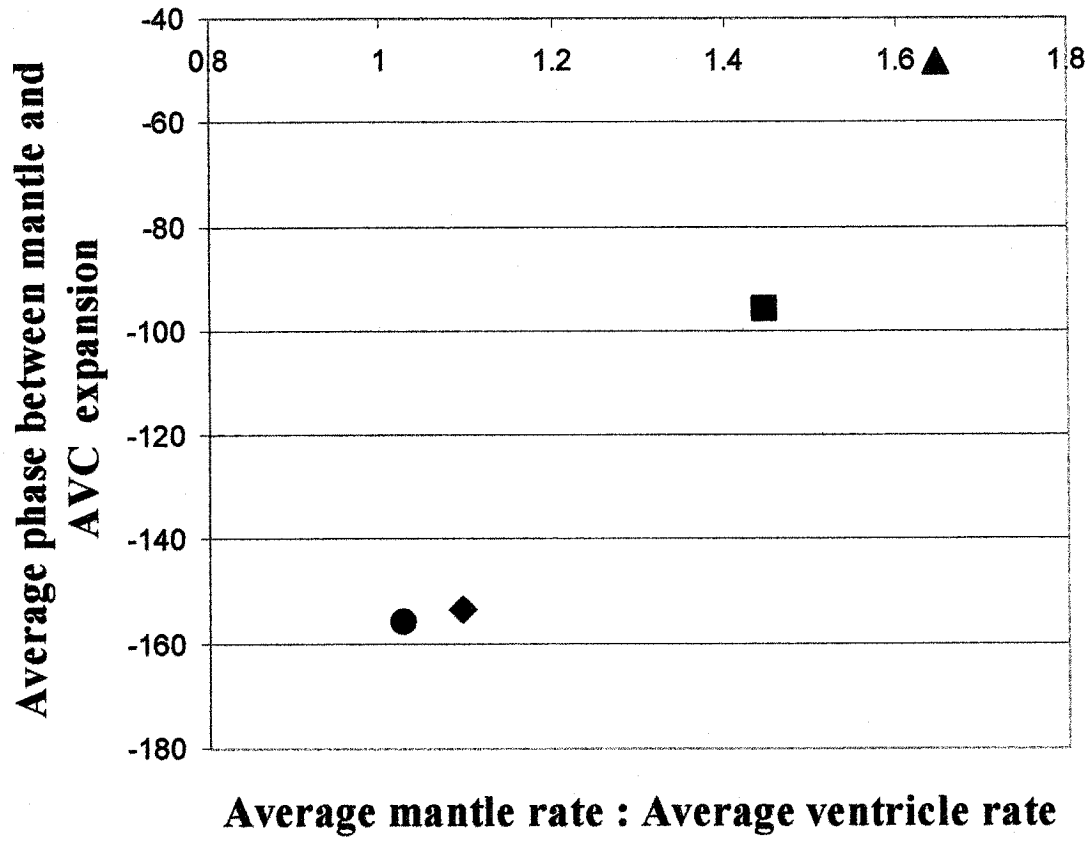


Figure 2.7. The phase shift between the mantle and the anterior vena cava (AVC) plotted against the ventilation rate : heart rate ratio. Fig. 2.6. shows the variation in the phase shift between mantle and AVC contractions between animals. However, the variation in the phase between animals is largely explained by how many times the mantle contracts per heart contraction (r^2=0.98, P=0.0082). Each symbol represents averaged data from one animal.

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 0.04-0.1m/s, slightly slower on average than the 0.1 m/s 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 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). By regulating flow into the lateral venae cavae, the Wells valve could also 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 contracted, 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 had the same frequency as ventricular contractions, but 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; in cuttlefish there is a valve separating the efferent branchial vessel and auricle (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; Skramlik, 1929; as cited in Johansen & Martin 1962; Wells and Smith, 1987). The nervous system might play 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 the other interconnected organs (Wells and Smith, 1987). Our results raise the possibility that such a region might instead be in the branch point (Fig. 2.4.).

Both heart rate and ventilation rate decreased with decreasing temperature. Adapted Q_{10}'s 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 (Frank 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. However, even if contractions of the anterior vena cava are timed to pressure increases within the mantle cavity, 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 affects 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.

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Chapter 3: Ventilatory, cardiac, chromatic and postural reactions to sudden visual stimuli in the cuttlefish *Sepia officinalis* Linnaeus

3.1. Linking information for this chapter

In the last chapter, I investigated circulatory and ventilatory function in resting cuttlefish. In this chapter, I investigate how ventilation rate, heart rate and behaviour change during an elevated behavioural state in cuttlefish. The elevated behavioural state is induced by the presentation of a sudden visual stimulus. This chapter is in preparation for submission to the Journal of Experimental Biology. It is co-authored by Shelley Adamo. For this chapter, I designed and developed the experimental protocol and experimental set-up, performed the experiments, developed and performed the analysis and wrote the manuscript.

3.2. Summary

We used non-invasive ultrasound imaging to monitor ventilatory, cardiac and postural responses of cuttlefish to sudden visual stimuli. Simultaneously we recorded behaviour using an overhead video camera. The cuttlefish response to sudden stimuli had four locomotory stages: pre-stillness, stillness, recovery from stillness and active swimming. Cuttlefish decreased their ventilation rate and heart rate during the response, the largest decreases occurring in cuttlefish with the lowest resting rates. Cuttlefish also suddenly changed colour, producing varying intensities of the chromatic and textural components of the Deimatic Display on their skin. Hyperinflation of the mantle

accompanied the response. Hyperinflation of the mantle was associated in time and magnitude with decreasing heart rate. Decreased heart rate may be a product of the unusual arrangement of the cuttlefish peripheral vasculature. Interestingly, the number of chromatic and textural components of the Deimatic Display present during a trial was not correlated with the magnitude of the decreases in ventilation rate, heart rate or the magnitude of hyperinflation.

The cuttlefish response to sudden visual stimuli is unlikely to increase crypsis because cuttlefish suddenly adopted the contrasting chromatic components of the Deimatic Display. The response may however increase cuttlefish stillness thereby increasing the ability of cuttlefish to attend to external stimuli. The cuttlefish mantle also filled with water during the response. Both increased attention and mantle filling may be important in helping cuttlefish prepare for possible flight by jet propulsion.

3.3. Introduction

Increased ventilation rate and cardiac output after a sudden stimulus has been well-studied (Wingfield, 2003). However, animals across many taxonomic groups appear to have an alternate response to sudden stimuli that involves decreasing ventilation rate and cardiac output well as behavioural freezing (Table 3.1.). 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 response; its heart rate increases, and it fights. Conversely, 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.

Table 3.1. 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

An alternate response can be elicited in the cephalopods *Octopus vulgaris* (Wells, 1980; Wells et al., 1987) and *Sepia officinalis* (Chichery, 1980) by rapidly approaching objects. On a behavioural level, these stimuli typically elicit the chromatic, textural postural and locomotory components of the Deimatic Display (Fig. 3.1.), including behavioural freezing (Hanlon and Messenger, 1996). The cephalopod Deimatic Display is part of a larger category of behaviour called deimatic behaviour. Deimatic behaviour occurs after a hiding cephalopod notices that a predator is approaching it, and before the cephalopod flees by powerfully forcing water from its mantle (jetting). It is defined 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).

In addition to the behavioural response, *O. vulgaris* decreases its ventilation rate and heart rate when presented with sudden visual stimuli (Wells, 1980; Wells et al., 1987). *S. officinalis* also decreases its ventilation rate during the Deimatic Display, although its heart rate has been found to remain stable (Chichery, 1980). Interestingly, studies monitoring the cardiac and ventilatory function of squid never report the Deimatic Display, or accompanying decreases in ventilation rate and heart rate (Wells et al., 1988; Shadwick et al., 1990). Unlike the behavioural response, the purpose of the cardiac and ventilatory responses of *Octopus* and cuttlefish during the Deimatic Display is less established. It is unclear why heart rate falls in *Octopus* and not in cuttlefish. Moreover that ventilation rate or heart rate should fall at all is surprising. Coleoid cephalopods remove almost all the oxygen from their blood during its circuit through the body

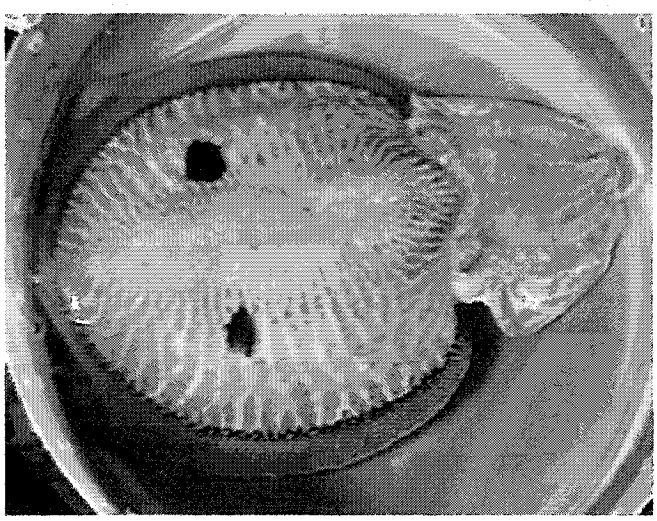


Figure 3.1. An example of the Deimatic Display seen in our experiments. This animal showed paired Mantle spots, dark elements on the fins, Mantle smoothness, Mantle widening and Arm widening. It did not show pronounced mantle paling.

(Withers, 1992). If circulation or oxygen exchange should slow or stop, they will be forced into anaerobic metabolism. Given that *Octopus* escape jetting is likely anaerobic (O'Dor and Webber, 1991) and limited by the accumulation of oxygen debt (Wells et al., 1987), it seems maladaptive to start accumulating oxygen debt before escape jetting has started.

Even though it appears as maladaptive in other animals as it is in cephalopods, the prevalence of an alternate reaction to threatening stimuli across vertebrate and invertebrate groups has made many theorize that it has a universally adaptive function. Four major theories explaining the alternate response have arisen from different branches of the vertebrate literature. They are summarized in Table 3.2. Unfortunately, because many vertebrate studies only monitor some 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.

In contrast to the vertebrate literature, which strives to lump all alternate responses together, the crustacean literature recognizes two types of alternate response: the startle response and the orientation response. The startle response is a violent visceral and motor response that includes cardiac arrest, and which habituates quickly. It may overlay the orientation response, whose primary goal is to focus sensory organs on new stimuli in order to gather more information. The orientation response includes bradycardia and habituates slowly (Cuadras, 1981). For simplicity, all reactions to sudden stimuli that include decreased cardiac function, decreased ventilatory function or behavioural freezing will be called an "alternate" response in this thesis. This terminology was chosen so as not to assume function or to ally responses with existing terminology before such

Table 3.2. Hypotheses from the vertebrate literature explaining lowered ventilation rate and heart rate after sudden stimuli.

Hypotheses	Reference
Decreased heart rate, ventilation rate and behavioural freezing reduce movement and noise from the animal. This helps it hide from predators.	Jacobsen, 1979 Barham et al,. 1985
Heart rate and ventilation rate slow because of lowered metabolism. This allows diving and borrowing air-breathing vertebrates to remain hidden underwater or in a hypoxic den for longer.	Smith & Woodruff, 1980 Smith et al., 1981
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 & Austin, 1981
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 & Hoffmann, 2002 Cooke et al., 2003

connections have been established. Until simultaneous heart rate, ventilation rate and behaviour recordings have been studied in vertebrates and molluscs, it will not be clear whether different functional forms of this response exist outside the crustaceans.

As previously demonstrated (chapter 2), we can monitor cardiac and ventilatory function in cuttlefish non-invasively using ultrasound. Simultaneously, we can monitor behaviour using an overhead video camera. The goal of this study was to quantify the behavioural, cardiac and ventilatory responses of cuttlefish to sudden stimuli non-invasively. By looking at many aspects of the response simultaneously, we hoped to better understand its purpose in cuttlefish. We also hoped to assess whether using non-invasive techniques might be important when studying the alternate response in cephalopods.

3.4. Methods

3.4.1. General housing conditions and experimental set-up

Cuttlefish were housed as described previously (chapter 2). Briefly, we obtained six juvenile, cultured *S. officinalis* Linnaeus 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 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.

As described in chapter 2, 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 images

(sonograms) were recorded on Hi-8 videotape. 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 inner and outer compartments allowed the transducer to be operated without disturbing the cuttlefish. 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 an opaque plastic curtain surrounded the experimental tank. A camcorder (CCD-TR910 NTSC, Sony) 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 behaviour videos are part of additional material accompanying this thesis (Videos 3.1. to 3.3.).

Cuttlefish were allowed to acclimatize to the experimental tank for at least two hours before being exposed to the startling visual stimulus. Several trials, including both the 2-h acclimatization and one presentation of the stimulus, were performed on each cuttlefish. Trials on a given cuttlefish were separated by at least two days.

3.4.2. 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). We do not know whether stressed cuttlefish, showing 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 reached a resting plateau during the 2-h acclimatization, we monitored these parameters over time for each trial.

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 30-s assessment. A behaviour score of 2 denoted that the cuttlefish was moving around the tank using its fins for at least part of the assessment period. A behaviour score of 3 denoted that the cuttlefish was moving around the tank using strong jets for at least part of the assessment period.

Then, using ultrasound, we estimated the ventilation rate and the heart rate of the cuttlefish. 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 obviously even during resting ventilation. The mantle does not (Bone et al., 1994). The first estimation of ventilation rate and heart rate was attempted 30 min after the cuttlefish had been transferred to the experimental tank because, before then, cuttlefish moved too much to

obtain the required ultrasound images. Subsequent estimations were made every 15 minutes, the 7th and last reading starting 2 h after the transfer. If estimations of physiological rates were not successful within 5 min, the attempt was stopped. Estimations were not always successful between minute 30 and minute 60 because of cuttlefish movement. Estimations were always successful at minute 120 and were averaged together for each cuttlefish separately. Then the average estimated rate was determined by averaging the resting rates over all cuttlefish.

To look for a resting plateau, we plotted behaviour, ventilation rate and heart rate for each time point listed above during the two-hour acclamation period. Several trials were performed on the same cuttlefish. For behaviour, we first calculated the median behaviour score for each cuttlefish separately for each time point. Then we calculated one median value for all cuttlefish for each time point. For ventilation rate and heart rate, we first calculated the rate difference (over a period of 10 s) between subsequent readings. We took the difference because absolute rates varied between cuttlefish and could not be meaningfully compared. These differences were then treated like the behaviour scores, above, resulting in one median value for all cuttlefish for each time point. A score of zero indicated no change from the last time point, and therefore that the ventilation rate or heart rate was stable.

3.4.3. Introduction of the startling visual stimulus

At least two hours after transfer to the experimental tank, we exposed cuttlefish to a sudden visual stimulus. The stimulus was a white bird-shaped Styrofoam cutout. The stimulus was moved at approximately 0.7 m/s over the experimental tank by a pulley system and left over the tank without further manipulation until the end of the trial. The

end of the trial was signaled by the cuttlefish settling on the bottom of the tank, and its ventilation rate and heart rate returning to approximately resting values (estimated over 10 s as in section 3.4.2.). 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 (Johansen and Martin, 1962; Wells, 1979; Chichery, 1980; Wells, 1980). It probably is important that the stimulus is consistent between trials; previous researchers have found that different visual and chemical stimuli can elicit different degrees of ventilatory response (Boyle, 1986; Boal and Ni, 1996; Boal and Golden, 1999). The stimulus was presented only once during each trial.

3.4.4. Quantification of ventilation rate and heart rate before and after presentation of the startling visual stimulus

We used ultrasound to visualize either movements of the collar flaps (ventilation rate) or ventricular contractions (heart rate; Fig. 1.2.: V) 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 anterior vena cava (Fig. 1.2.: AVC), and when visualizing the ventricle, we could see the lateral venae cavae (Fig. 1.2.: LVC; both the AVC and LVC are indicators of venous return). It was impossible to reliably capture 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 captured at a time.

If the cuttlefish moved during stimulus presentation, 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 was successfully collected from the collar flaps (ventilation) in one trial for each of the six cuttlefish. Systemic heart data was 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 recorded the times (±1/15 s) at which the collar flaps were closest to the midline, from successful sonograms of the collar flaps. 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 these times to calculate the instantaneous rate per minute for each contraction using the following equation:

Equation 3.1:

$$\text{Instantaneous rate} = (\text{period of contraction})^{-1} * 60;$$

where the period of contraction is measured in seconds.

These instantaneous rate calculations were separate from the estimates of ventilation rate and heart rate made during acclimatization (section 3.4.2). To determine whether instantaneous rates were lower after presentation of the stimulus, we found the lowest rate after the stimulus using recent procedures of visual inspection (Ide and Hoffmann, 2002; Cooke et al., 2003). We stipulated that individual instantaneous rates after the stimulus had to differ from all individual instantaneous 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 instantaneous rate before the stimulus and lowered it by the measurement error (±1/15 s). If any of the 7 instantaneous rates after 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 instantaneous rate before the stimulus and increased it by the measurement error (±1/15 s). If any of the 7 instantaneous rates after the stimulus were higher than this, the rate was considered to have increased. In some trials, the instantaneous rates both increased above and decreased below resting levels after the stimulus. As a baseline for each trial, we averaged together the instantaneous rates for the entire 30-s resting period before the stimulus. This measurement was more precise than the average estimated rates calculated during acclimatization. The magnitude of increases or decreases in rates were calculated as a percentage of the baseline rate.

3.4.5. Quantification of the chromatic, textural and postural responses to the startling visual stimulus

Behavioural data was obtained for all 26 trials, and displayed considerable variability. The behavioural reaction during the alternate response was best described as the Deimatic Display. Variability in the Deimatic Display has been noted by previous authors (Chichery, 1980; Hanlon and Messenger, 1996) who also identified its key components: Mantle paling, paired Mantle spots (that look like eyes), Mantle smoothing, dark fin elements, Mantle flattening, Fin stalling (i.e. the fins stop moving) and spreading, Arm spreading, Pupil dilation, and a Dark ring around the eye (Fig. 3.1.). We did not observe the Dark eye ring in our experiments. During Pupil dilation, previous authors

describe pupils that are fully dilated. Pupil dilation in our experiments was always weak, and never more than half the maximal size. In order to quantify the chromatic and textural reaction to the stimulus, we selected the four key components of the display that both differed obviously between our trials (i.e. not pupil dilation or dark eye ring), and were not postural (i.e. not flattening, spreading or fin stalling). We wished to look at posture separately. The chosen components were Mantle paling, Mantle spots, 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.

Previously described Mantle flattening implies both a lateral widening of the mantle and a reduction in dorso-ventral height. To quantify Mantle flattening and Arm spreading, we used the public domain NIH Image program (version 1.62, developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/) to measure the structures described below and depicted in Figure 3.2.

From the behavioural video:

a. the width of the mantle at its widest point (this was approximately halfway down and designated the "mid-mantle").

b. the distance between the outer edges of the base of the 4th arms.

From the ultrasound video 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 anterior mantle cavity, excluding viscera (one side only).

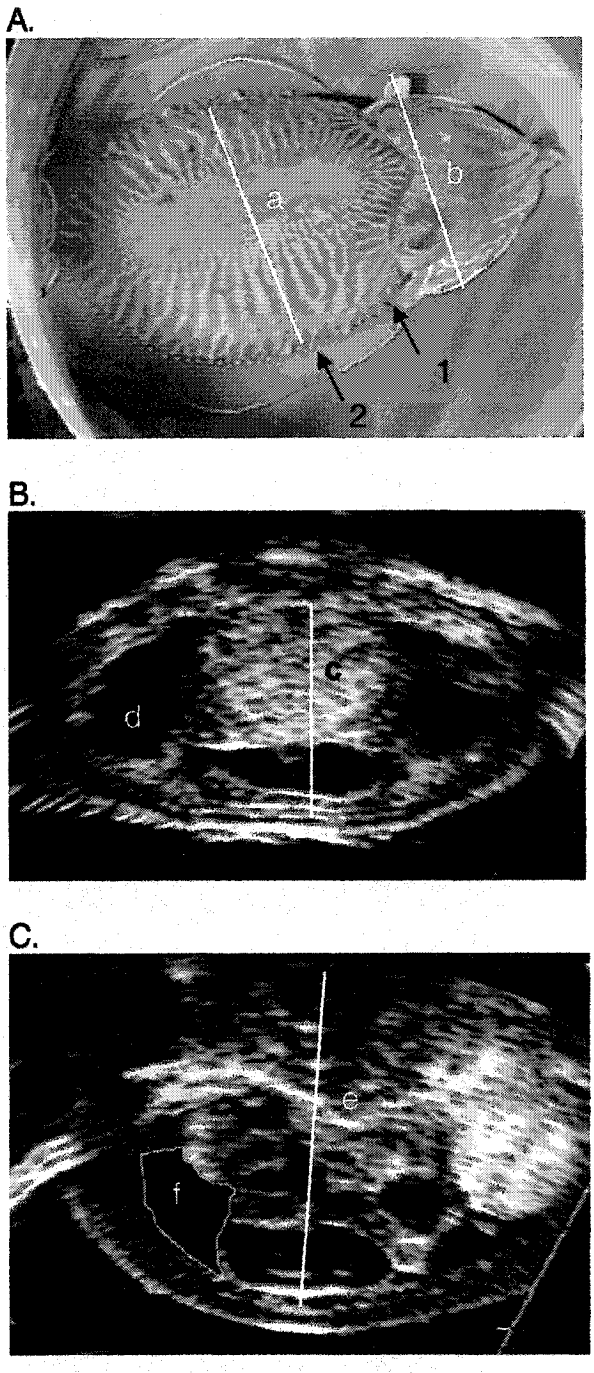


Figure 3.2. The parameters measured to quantify posture. A. The width of the mantle was measured along line (a), the width of the arms along line (b). B. A sonogram taken along plane 1 in A. The dorsal mantle is at the top and ventral mantle at the bottom. The dorso-ventral height of the anterior mantle was measured along line (c). The area of the anterior mantle cavity is space (d). C. A sonogram taken along plane 2 in A. The orientation is as in B. The dorso-ventral height of the mid-mantle was measured along line (e). The cuttlebone is not visible in this sonogram, so dorsal end of line is arbitrary. Measurements would not have been taken in this trial. The area of the mid-mantle cavity is space (f).

From ultrasound video 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 mid-mantle cavity, excluding the viscera (one side, from the midline).

For each trial, we captured one frame on an inhalation within 5 s before the stimulus and one after the stimulus at the onset of the most intense part of the behavioural reaction (stage 2, or when structure appeared most changed, see results). The frame was measured three times and the mean and the standard deviation taken. The mean was the reported value. The standard deviation was taken as the measurement error. If measurements before the stimulus and those after did not differ by more than the measurement error, there was considered to be no change. If the cuttlefish changed orientation after the stimulus, the trial was omitted. If more than one set of measurements was obtained for a cuttlefish, the mean was taken. 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). The response of the fins was not analyzed.

3.5. Results

3.5.1. Behaviour, ventilation rate and heart rate during acclimatization

Immediately after transfer, cuttlefish swam agitatedly around the experimental tank. However, from minute 75 of the acclimatization onward, cuttlefish showed a median

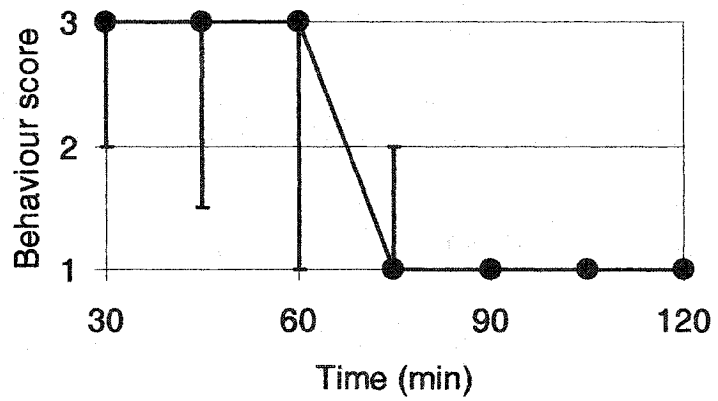
behaviour score of 1, indicating that cuttlefish were resting quietly on the bottom of the experimental tank (Fig. 3.3.A.). After the 2-hour acclimatization, in the 30-s period before the stimulus, cuttlefish showed resting body colouration. The important components are summarized in Table 3.3.

During acclimatization, median ventilation rate did not drop between the first reading at minute 30 and the last reading at minute 120 (Fig. 3.3.B.). The average estimated ventilation rate at minute 120 was 30.3 ± 6.6 breaths/min (6 cuttlefish). Median heart rate decreased until minute 90 (as evidenced by negative values), and then became stable (Fig. 3.3.C.). The average estimated heart rate at minute 120 was 22.0 ± 2.5 beats/min (6 cuttlefish).

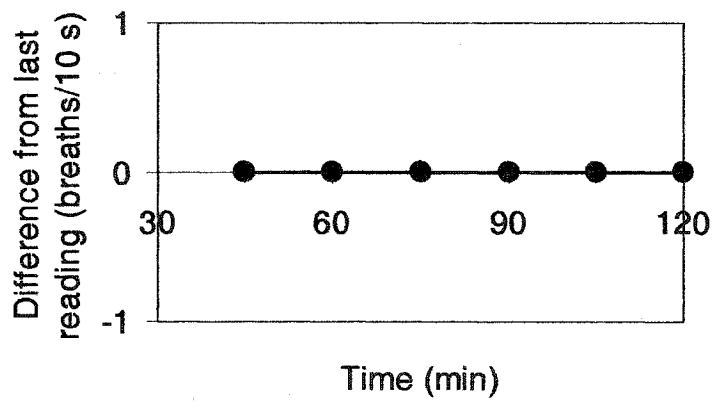
3.5.2. The four locomotory stages of the reaction

After minute 120, the cuttlefish were exposed to a sudden visual stimulus. Cuttlefish typically had a four stage reaction to the stimulus that was defined by locomotory characteristics (Video 3.1). These stages are summarized in Table 3.4., and described below. The first and shortest stage (duration range: 1-12 s) started with the onset of the behavioural response, and 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 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: 7-332 s). This usually started with ventilation and the resulting movements of the mantle, fins and of the lateral ridges on the 4th arms. Movements usually became progressively larger until the cuttlefish started swimming at the start of the fourth stage. The fourth stage ended when

A.



B.



C.

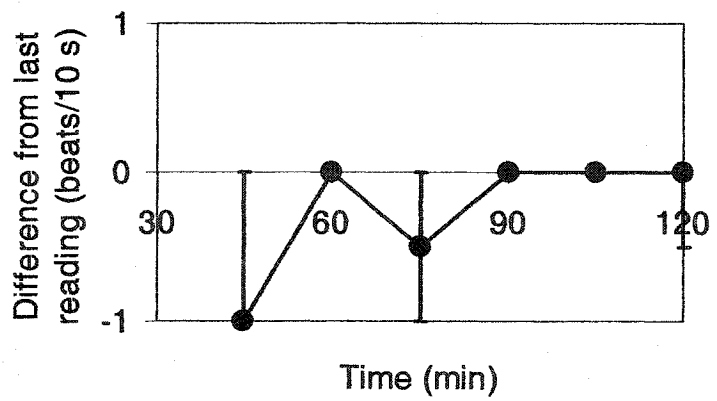


Figure 3.3. A. The median behaviour scores (± first and third quartiles) during the 2 h acclamation period. A score of 1 indicates resting cuttlefish B. Median difference between subsequent estimations of ventilation rate (± first and third quartiles) during the two hour acclamation period. A score of 0 indicates no change between readings. C. Same as B., but for heart rate.

Table 3.3. The colouration of female and male cuttlefish in the experimental tank after at least 2 h, and before presentation of the sudden visual stimulus.

Component of body pattern	Occurrence in females		Occurrence in males			
	#11	#17	#12	#18	#22	#26
Beige mantle	6/6	4/4	2/4	2/3	0/4	0/5
Medium intensity Zebra striping	0/6	0/4	2/4	1/3	4/4	4/5
Papillae erected on mantle at fin margin	4/6	3/4	3/4	3/3	3/4	2/5
Papillae grading up mantle	6/6	4/4	3/4	3/3	2/4	3/5
White fin spots	6/6	4/4	3/4	3/3	4/4	5/5
White line along fin edge	6/6	4/4	4/4	3/3	4/4	5/5
Light, translucent fins	6/6	4/4	3/4	3/3	3/4	3/5
Fins resting out to the sides	5/6	3/4	4/4	3/3	4/4	2/5
Stationary fins	4/6	3/4	0/4	0/3	1/4	0/5

Table 3.4. A summary of the four stages of the alternate reaction in cuttlefish. They are defined by locomotion. Duration is expressed as the median over all cuttlefish (first, third quartiles). Also included are the important ventilation rate, heart rate, and chromatic and textural changes.

	Stage 1	**Stage 2**	**Stage 3**	**Stage 4**	**End of experiment**
Defining locomotion	Slowing of movements	Onset of behavioural freezing	Resumption of movement	Onset of 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 (Table 3.5.)	Heart rate decreased below resting levels	Ventilation rate decreased below resting levels	Ventilation rate and heart rate recovered	None	Ventilation rate and heart rate returned to resting
Important chromatic and textural events	Rapid chromatic change	Few chromatic changes; Deimatic Display	None	None	None

the cuttlefish again settled on the bottom of the experimental tank and its heart and ventilation rates returned approximately to resting values. This stage took between 3 and 28 min. All six cuttlefish typically showed this four-stage pattern (20/26 trials). Four of the six cuttlefish occasionally skipped stages. 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 both skipped stage 4 1/4 trials).

3.5.3. Ventilatory and cardiac reactions to sudden visual stimuli

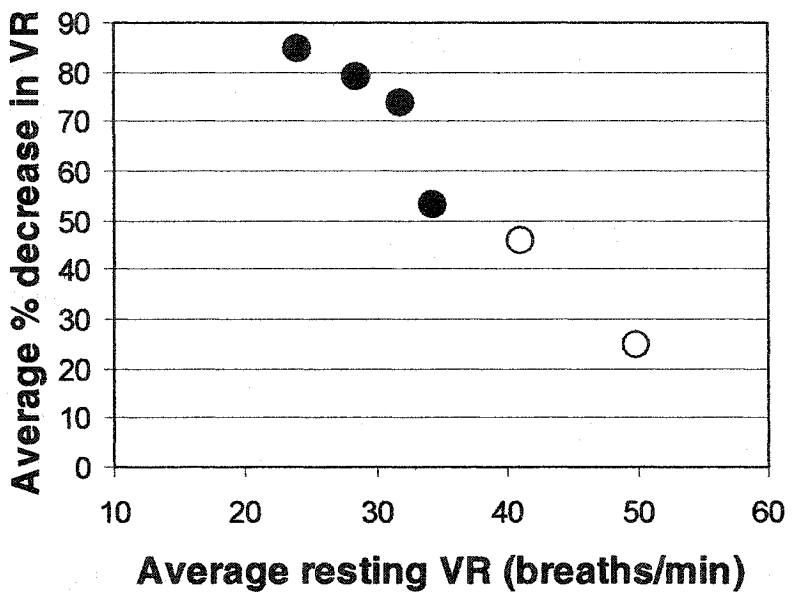
From the sonograms that were recorded simultaneously with the behaviour video, we quantified how ventilation rate (Video 3.2.) and heart rate (Video 3.3.) changed after presentation of the visual stimulus. We also monitored venous contractions of the anterior vena cava and lateral venae cavae on the sonograms. Because sonograms and behavioural video were synchronized by an audio cue, we were able to determine when the changes in ventilation rate and heart rate occurred relative to the four reaction stages.

After presentation of the stimulus, ventilation rate always decreased below resting rates, either in stage 2 or at the onset of stage 3 (Table 3.5.). Ventilatory movements of the collar flaps not only slowed, but stopped entirely in 4/6 cuttlefish for 3.7-16.3 s. The percent decrease in the instantaneous ventilation rate was inversely proportional to the resting baseline ventilation rate of the cuttlefish (Linear regression: r^2=0.95, P=0.0009, N=6; Fig. 3.4.A.). In other words, the lower the rate before the stimulus, the larger the percent drop in rate after the stimulus. Before decreasing, ventilation rate increased above resting for one breath in 3/6 cuttlefish (Fig. 3.5.A.). These were not always the trials that subsequently showed ventilatory arrest.

Table 3.5. Summary information about the ventilatory and cardiac responses of cuttlefish to sudden stimuli. Only data from trials including all reaction stages are included.

	# of trials # of cuttlefish	Rate decrease Median (1^{st}, 3^{rd} quartiles)	Stage of decrease	Occurrence of arrest	Duration of arrest (range, s)	Occurrence of rate increases
Ventilation rate	6 trials 6 cuttlefish	63.5% (46.0%, 78.9%)	Stage 2: 4/6 cuttlefish Onset of stage 3: 2/6 cuttlefish	4/6 trials	3.7-16.3 s	3/6
Heart rate	7 trials 5 cuttlefish	48.3% (20.0%, 66.5%)	Stage 1: 4/5 cuttlefish, 5/5 trials Stage 2: 1 cuttlefish, 2/2 trials	3/7 trials 2/5 cuttlefish	5.3-20.0 s	2/7 trials 2/5 cuttlefish

A.



B.

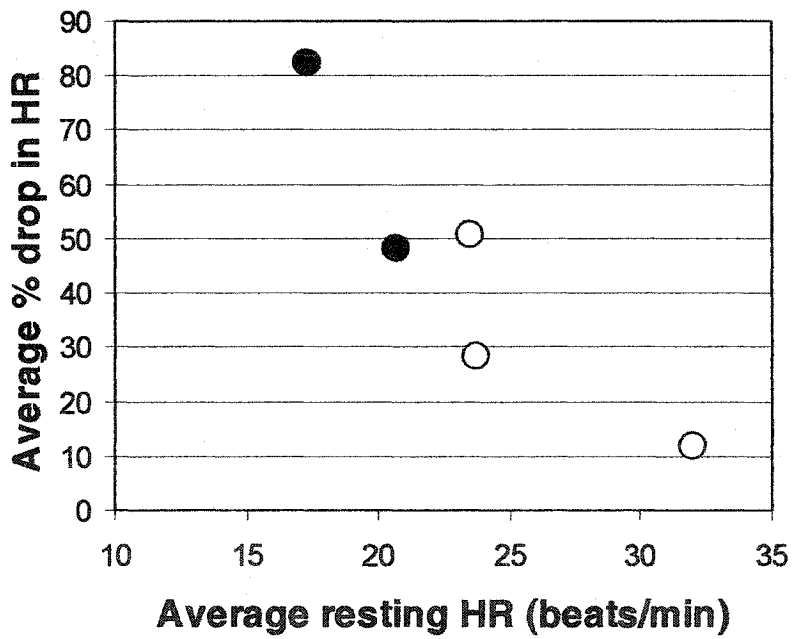


Figure 3.4. A. The relationship between resting ventilation rate (VR) and the percent decrease in ventilation rate after the stimulus. Open circles: ventilatory slowing; closed circles: ventilatory arrest. B. Same as A., but for heart rate (HR). Open circles: cardiac slowing; closed circles: cardiac arrest. For both, faster rates show less of a drop and are less likely to experience arrest. Each point is the averaged data for one animal.

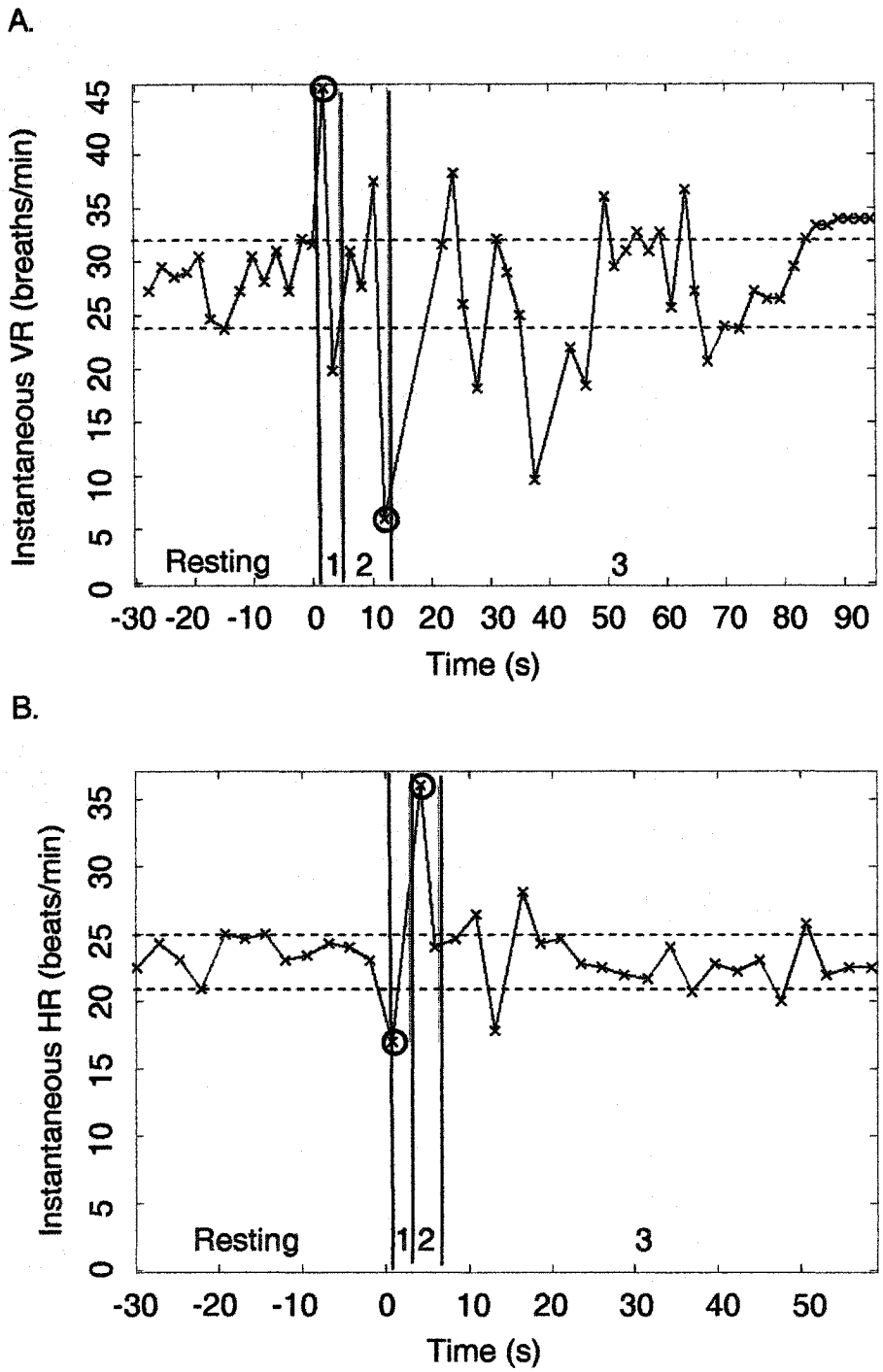


Figure 3.5. Representative examples of the ventilatory (A) and cardiac (B) reactions to startling stimuli. The stimulus was presented at time 0. Vertical lines delineate resting behaviours and reactions stages 1-3. Dashed lines delimit the maximum and minimum instantaneous rates before the stimulus. Circles emphasize maximum and minimum instantaneous rates after the stimulus. A. Cuttlefish #12: Decrease in ventilation rate: 78.9%. B. Cuttlefish #22: Decrease in heart rate: 28.3%.

In resting cuttlefish, the contractions of the anterior vena cava share the rate of ventilatory contractions (chapter 2). 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).

If all reaction stages were present, heart rate decreased after presentation of the sudden stimulus, typically in stage 1 (Table 3.5.). 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%, 1/3 trials, 1/3 cuttlefish). Cardiac arrest occurred in 3/7 trials that included all reaction stages (2/5 cuttlefish) and could last for 5.3-20.0 s. As with ventilation rate, the percent decrease in heart rate was inversely proportional to the resting rate of the cuttlefish (Linear regression: r^2=0.83, P=0.031, N=5; Fig. 3.4.B). Heart rate occasionally increased above resting (2/7 trials, 2/7 cuttlefish) for one contraction immediately after the lowest instantaneous heart rate (Fig. 3.5.B.).

In resting cuttlefish, contractions of the lateral venae cavae share the contraction rate of the ventricle (chapter 2). We observed that, 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).

3.5.4. Chromatic and textural reactions to sudden stimuli

Upon presentation of the stimulus, all cuttlefish showed a chromatic and textural reaction, although its components varied in duration and intensity both between trials and between animals (e.g. Video 3.1.). The cuttlefish showed all of the chromatic and textural components of the Deimatic Display described by previous authors except the

dark eye ring. Unlike previous authors, we never saw complete pupil dilation, and during 21/26 trials, there was no obvious dilation at all. This may have been because the experimental tank was much brighter than the cuttlefish's usual home tanks.

To place the chromatic and textural reaction in a temporal framework, we investigated the stages at which different components of the chromatic index appeared. Most chromatic and textural components started in stage 1 of the reaction (Table 3.6.). Cuttlefish #22 showed unusual responses. Mantle paling is one of the key components of the Deimatic Display, and all but cuttlefish #22 showed it. Cuttlefish #22 did not become beige or white during the reaction, but instead darkened in 2/4 trials.

To assess the connection between the chromatic and the ventilatory and cardiac reactions, we tested whether the chromatic reactions were dependant on the same things as the physiological reactions, i.e. the presence of all reaction stages and resting physiological rates. Behavioural changes were quantified by the chromatic and textural index. Indices ranged from 1-4 both when cuttlefish showed all reaction stages (20 trials) and when they skipped reaction stages (6 trials). The most intense chromatic and textural reactions were possible even when reaction stages were missing. When the chromatic and textural index was compared to resting ventilation rate and resting heart rate, there was no correlation (Fig. 3.6.A, B). Unlike physiological responses to sudden stimuli, chromatic and textural responses were not tied to resting physiological rates.

Table 3.6. The onset of the chromatic and textural elements of Deimatic during reaction stages 1-3. Only trials that showed all reactions stages are included. Trials with chromatic changes are bolded to make them more evident. A. Stage 1 (pre-stillness). B. Stage 2 (stillness). C. Stage 3 (recovery from stillness).

A.

Animal Stage 1	Mantle spots initiated	Mantle paler than previous stage	Fins darker than previous stage	Smooth mantle
11	0/4	**4/4**	**3/4**	0/4
12	**2/2**	**1/2**	**2/2**	0/2
17	**2/3**	**2/3**	**3/3**	0/3
18	**3/3**	**2/3**	**3/3**	**2/3**
22	0/3	0/3	**2/3**	0/3
26	0/5	**5/5**	**5/5**	0/5

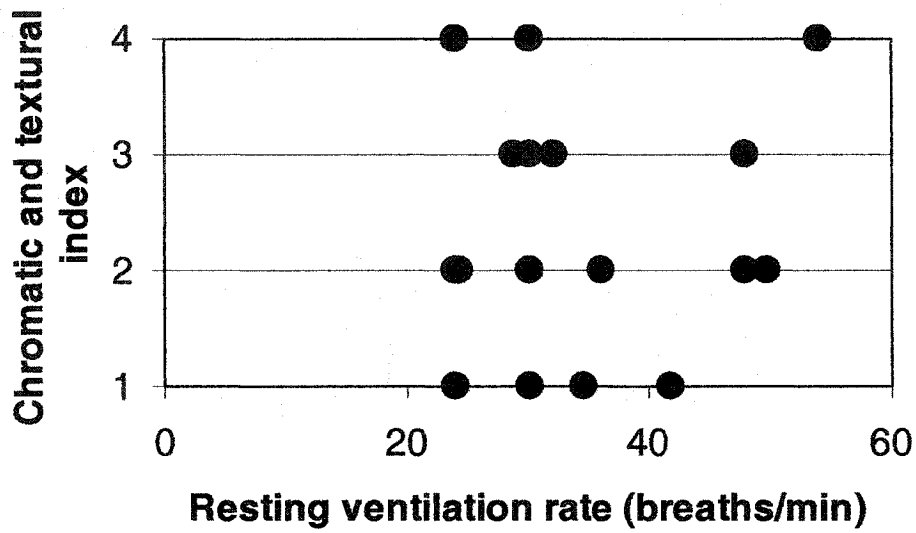
B.

Animal Stage 2	Mantle spots initiated	Mantle paler than previous stage	Fins darker than previous stage	Smooth mantle
11	0/4	0/4	0/4	0/4
12	0/2	0/2	**1/2**	0/2
17	0/3	**1/3**	0/3	**1/3**
18	0/3	0/3	0/3	**3/3**
22	0/3	0/3	0/3	**1/3**
26	0/5	0/5	0/4	0/5

C.

Animal Stage 3	Mantle spots initiated	Mantle paler than previous stage	Fins darker than previous stage	Smooth mantle
11	**1/4**	0/4	0/4	0/4
12	0/2	0/2	0/2	0/2
17	0/3	0/3	0/3	**1/3**
18	0/3	0/3	0/3	**2/3**
22	0/3	**1/3**	0/3	**1/3**
26	0/5	0/5	0/4	0/5

A.



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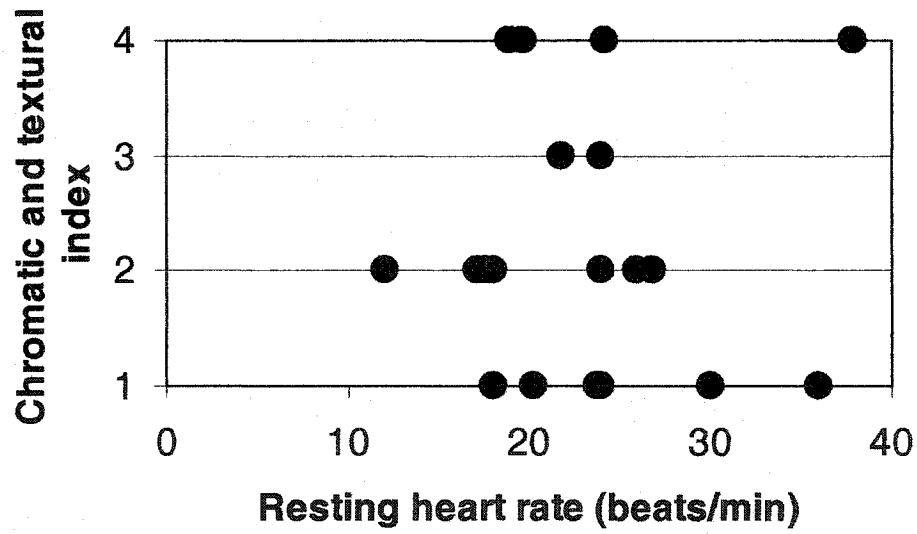

Figure 3.6. A. The relationship between resting ventilation rate and the chromatic and textural index. B. The relationship between resting heart rate and the chromatic and textural index. The resting rate was the baseline rate for the given day. Data are not averaged within an animal.

3.5.5. Postural responses to sudden visual stimuli

Besides locomotory, ventilatory, circulatory and chromatic changes, cuttlefish also changed their shape after seeing a sudden stimulus. We quantified changes in the shape of the anterior mantle (e.g. Video 3.2.) and the mid-mantle (e.g. Video 3.3.) and compared it to the other changes.

Measurements from the behavioural video revealed that the mid-mantle and the arms both widened significantly during the cuttlefish alternate response (Table 3.7.). Measurement of anterior mantle depth was possible only from sonograms of the collar flaps of 4/6 cuttlefish. In these, the anterior mantle always became shallower after the stimulus (4/4 cuttlefish, Table 3.7.). In 3/6 cuttlefish, we were able to measure the cross-sectional area of the mantle cavity beside the neck. This always increased (3/3 cuttlefish, Table 3.7.). Measurements from the sonograms of the systemic heart unexpectedly revealed that the mid-mantle became deeper after the stimulus in 4/6 cuttlefish. In the other two cuttlefish, it became shallower. We measured the area, excluding the viscera, of the mid-mantle cavity in 5/6 cuttlefish. This increased, often dramatically, in 4/5 cuttlefish (Table 3.7.). Given the high variability in area increases (range: 0-175.3%) and the small sample size, 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 mid-mantle. To facilitate discussion, the increase in area around the mid-mantle viscera will be called hyperinflation, because the area is larger than the mantle inflation seen during normal ventilation. To understand the role that changes in mantle shape might play in this reaction, we compared the degree of hyperinflation to other aspects of the response

Table 3.7. The percent change in the postural parameters measured before and after presentation of the stimulus (see Fig. 3.2. for measured parameters). Measurements that were larger after presentation are positive, those that were smaller are negative.

Postural parameter	**Median percent change (first, third quartile)**	**N**	**Paired-sample *t* test**
Mid-mantle width	11.3 (10.1, 12.6)	6	t=6.92 P<0.001
Arm width	15.6 (11.5, 19.6)	6	t=5.89 P<0.005
Anterior mantle depth	-18.5 (17.1, 20.4)	4	t=16.67 P<0.001
Anterior mantle cavity area	8.7 (8.3, 15.6)	3	t=4.58 P<0.05
Mid-mantle depth	4.9 (-5.7, 12.5)	6	t=1.20 NS
Mid-mantle cavity area	70.7 (3.2, 164.0)	5	t=2.236 P<0.10

(decreases in heart rate, the chromatic and textural index and the reaction stages). Percent hyperinflation was chosen because it was the most relevant descriptor of mid-mantle shape change.

Hyperinflation and decreased heart rate could be related. The magnitude of hyperinflation was related to percent decrease in heart rate, with borderline significance (Linear regression: r^2=0.74, P=0.062, N=5; Fig. 3.7.). Furthermore, maximum hyperinflation was typically associated with falling or minimum heart rates (6/8 trials, 4/5 cuttlefish; however 1 cuttlefish showed maximum hyperinflation before any response in heart rate in 2/2 trials).

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). Chromatic and textural changes are not related to ventilatory, cardiac or postural changes.

Similar to the magnitude of cardiac decreases, the magnitude of hyperinflation was greatest in trials with all reaction stages (Fig. 3.7).

3.6. Discussion

Many vertebrates and invertebrates decrease their ventilation rate and heart rate and exhibit behavioural freezing in response to sudden stimuli (see Table 3.1. and introduction for references). Although the cuttlefish response showed complexity and variability, it is evident that cuttlefish also decreased their heart rate and ventilation rate, and exhibited behavioural freezing after presentation of sudden visual stimuli. The

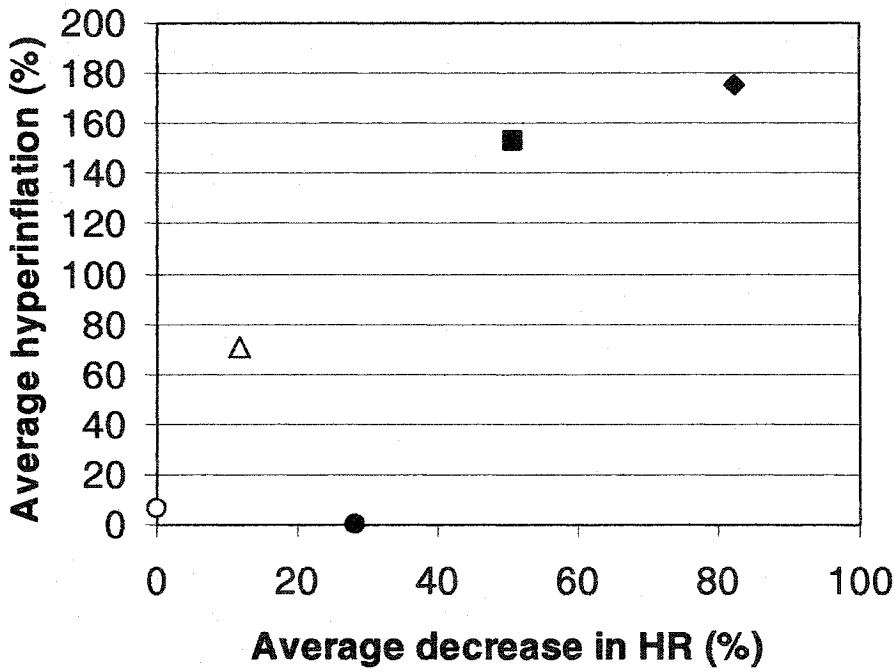

Figure 3.7. The relationship between the percent decrease in heart rate (HR) and the percent hyperinflation. Each point represents the averaged data of one animal. Open symbols indicate trials that missed reaction stages, closed symbols indicate trials with all reaction stages. The chromatic index is represented by the shape of the symbol: circle=1, diamond=2, triangle=3, square=4.

reaction was usually composed of four reaction stages, which are summarized in Table 3.4. The presence of all four reaction stages coincided with trials in which cuttlefish had the biggest decreases in heart rate and the most hyperinflation. However the presence of all reaction stages did not guarantee the maximal response of any single parameter, and was unrelated to the chromatic and textural response. All parameters showed a great variability in intensity, some of which can be explained by factors other than the presence of all the reaction stages.

The magnitude of the heart rate drop was determined by resting heart rate; lower resting heart rates being associated with a larger percent decrease in rate and higher resting heart rates being associated with lower percent drops. This is counterintuitive because higher rates have "further to fall" during the reaction (Law of Initial Values, Richards, 1980). But cuttlefish with high resting rates could have had high resting metabolic demands (e.g. during digestion, pathology or oogenesis) that precluded lowered oxygen distribution and consequently prohibited decreased heart rate and ventilation rate. Mid-mantle hyperinflation (postural change), in turn, was related to the percent decrease in heart rate. Given that the drop in heart rate was correlated to resting heart rate, the magnitude of hyperinflation may also have been closely related to the resting metabolism of the cuttlefish.

Interestingly, the magnitude of chromatic and textural changes (chromatic and textural index) was not related to the magnitude of postural changes or dependant on resting ventilation or heart rates. This is perhaps not surprising considering that the Deimatic Display, which describes the chromatic and textural responses shown by our cuttlefish, is believed to be an obvious signal sent by the cuttlefish to indicate to a potential predator that it has been seen (Hanlon and Messenger, 1996). Such anti-

predation signals need to be sent regardless of when the cuttlefish last ate, or whether it is about to lay eggs. Variability of the Deimatic Display is probably more dependant on what is appropriate to the external environment than on the metabolism of the cuttlefish.

While the purpose of the Deimatic Display is explained, the purpose of the ventilatory, cardiac and postural reactions is not immediately clear, especially considering that cuttlefish are considered to be oxygen-limited. It is unlikely that ventilation rate and heart rate decrease to reduce noise or to increase crypsis (Table 3.2.). Ventilation rate and heart rate decreased at different times, allowing either the mantle or the heart to always be 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 also unlikely that decreased ventilation rate and heart rate are due to decreased metabolic rate during the reaction (Table 3.2.). In fact, cuttlefish mantle muscle and head retractor muscles show increased activity during the alternate response (Chichery, 1980), very likely increasing metabolic demand.

Ventilation rate may however drop in order to keep the cuttlefish still. It is unusual that cuttlefish would become still while breaking crypsis, but it may be advantagous. Becoming suddenly still during the Deimatic Display may make subsequent flight by sudden jetting more surprising. Stillness may also increase the acuity of the cuttlefish's eyes and the lateral-line analogue on its arms, allowing the cuttlefish to gather more information about the approaching predator and potential escape routes.

After the sudden stimulus, ventilation always stopped at the same point in the ventilatory cycle. Chichery (1980) noted that ventilation usually stopped on an inhalation during the alternate response. Similarly, we found that cuttlefish almost always

hyperinflated their mantles. 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) persists (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 were not an effective deterrent to the predator.

A disadvantage of hyperinflation is that it might necessitate 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, as in mammals (Guyton, 1991), muscle contraction compresses intramuscular blood spaces. In cuttlefish, most capillaries run perpendicularly to the radial muscles (Bone et al., 1981), and therefore could be compressed by radial muscle contraction. During intense radial muscle contraction, as seen during jetting (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.

Further evidence supports the theory that blood vessels collapse during hyperinflation. First, our cuttlefish's anterior venae cavae and systemic hearts filled during 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 reaction (Johansen and Martin, 1962).

Third, whenever octopods jet, even in the absence of "psychological" disturbance, their hearts always stop (Johansen and Martin, 1962; Wells et al., 1987). When an octopus moves using its arms, heart rate increases, rather than decreases (Wells et al., 1987), suggesting that movement alone does not necessitate a drop in heart rate. Cardiac function appears to be interrupted by mantle thinning. Unlike vertebrates, octopods and cuttlefish might react with bradycardia both when they jet to flee and when they hyperinflate and become still. Interrupted cardiac function is not seen in jetting squid (Wells et al., 1988; Shadwick et al., 1990), however, they jet constantly and may prevent blood from being forced into the rest of the body during jetting by using muscular valves. These valves are located peripherally, where veins exit the mantle ("peripheral hearts", Williams, 1909). There is no mention of "peripheral hearts" in *Sepia* or in *Octopus*.

It is possible that increased aortic pressure during the alternate response caused a reflexive decrease in heart rate (Smith, 1981), but the larger part of cardiac inhibition is more likely mediated centrally by the visceral nerve. *In vitro* stimulation of the visceral nerve results in cardiac inhibition (Fry, 1909). In octopods, and possibly other coleoid cephalopods, the visceral nerve must be intact for the full cardiac response to occur during the alternate response (Wells, 1980). Furthermore, cardiac inhibition is too rapid for anything but neural mediation (Wells, 1980) and the contractions of other organs innervated by the visceral nerve (Smith and Boyle, 1983), such as the lateral venae cavae (this study) and the branchial hearts (Johansen and Martin, 1962) also stop simultaneously with the ventricle. Cardiac slowing and arrest in coleoids during the alternate reaction seem to be centrally and neurally mediated, as in crustaceans (Cuadras, 1981), some fish (Ide and Hoffmann, 2002) and some mammals (Causby and Smith, 1981). Central mediation, rather than simple reflex, is perhaps necessary in cephalopods

because the blood is returned to the systemic ventricle by the active contraction of the veins. Consequently, the heart may only stop pumping provided that the contractions of the upstream veins are also inhibited. For this, the integrated inhibition provided by the visceral nerve may be necessary.

Imaging ultrasound was useful, but not without limitations in this study. It was difficult to quantify the magnitude of the contractions (e.g. stroke volume or pressure produced) or to measure blood flow. The first made it difficult to determine when contractions had returned to resting strength. The second made it impossible to know whether cardiac arrest resulted in circulatory standstill. However, it is advantageous to use non-invasive techniques to study the ventilatory and cardiac responses of the cephalopod alternate reaction, given the inverse relationship between the resting rate and the magnitude of the physiological reaction. Our trials using ultrasound resulted in lower resting ventilation rates and heart rates than previously reported with some invasive techniques (Table 3.8.). It is possible that we observed decreased cardiac function during the alternate reaction while Chichery (1980) saw no change because of the elevated resting heart rate of his cuttlefish (45-50 beats/min at 13°C vs. my 22.0±2.5 beats/min 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 smaller than ours, rather than more stressed. Another explanation for the differences between our results and those of Chichery (1980) is that Chichery used the Deimatic Display, and not hyperinflation to determine whether his cuttlefish responded. Decreased heart rate was not related to the Deimatic Display in our trials, but instead was correlated with hyperinflation. Depending on his sample size, the

Table 3.8. The resting ventilation and heart rates reported in previous studies on *S. officinalis*. They are standardized to our temperature of 15°C using the Q_{10} values calculated in chapter 2. If size was reported in weight instead of mantle length, the equivalent mantle length was interpolated using values of Forsythe et al. (1994). It is not clear how to standardize for the effect of size on ventilation and heart rates, but larger animals are expected to have slower heart rates (Chichery and Chanelet, 1972).

Study	Parameter studied	Technique	Temperature (°C)	Cuttlefish size (mantle length in cm)	Reported rate	Standardized rate
Present study	Ventilation rate	Non-invasive (ultrasound)	15	15.5-18.5	30.3±6.6 breaths/min	30.3±6.6 breaths/min
Boal and Ni, 1996	Ventilation rate	Non-invasive (visual observation)	19-21	7.5-10.5	45 breaths/min	32.1 breaths/min
Mislin, 1966	Ventilation rate	Invasive (leads forced through mantle)	16-18	20-35	45 ± 9 breaths/min	39.3 breaths/min
Bone et al., 1994	Ventilation rate	Invasive (electrodes in mantle tissue)	18.5	5-25.0	48-60 breaths/min	42.6 breaths/min
Present study	Heart rate	Non-invasive (ultrasound)	15	15.5-18.5	22.0±2.5 beats/min	22.0±2.5 beats/min
Chichery and Chanelet, 1972	Heart rate	Invasive (EKG leads)	16	13.6-20.0	20-30 beats/min	22.8 beats/min
Mislin, 1966	Heart rate	Invasive (leads forced through cuttlebone)	16-18	20-35	43±9 beats/min	35.8 beats/min
Chichery, 1980	Heart rate	Invasive (EKG leads)	13	Not reported	45-50 beats/min	57.1 beats/min

examples of the Deimatic Display he saw may not have had simultaneous decreases in heart rate.

This is the first study to look simultaneously and non-invasively at the behavioural, ventilatory, cardiac and postural response of cuttlefish to startling stimuli. Considering our small sample size, and many post-hoc analyses, this study is only a beginning. Nevertheless, we have been able to demonstrate that cuttlefish decrease their ventilation rate and heart rate after sudden visual stimuli while hyperinflating their mantles and showing the Deimatic Display. Additionally, our data reveal links between resting metabolism, hyperinflation and decreased heart rate that can be explicitly tested. Overall, it is likely that physiological, behavioural and postural reactions of cuttlefish to sudden stimuli keep cuttlefish still for as long as resting metabolic rates will allow, while filling the mantle with water in anticipation of subsequent jetting. Stillness can make subsequent jetting more surprising to a would-be predator, or can improve sensory function. The alternate reaction in cuttlefish seems to be more in line with the crustacean orientation response than with any of the proposed functions from the vertebrate literature. Given that the behavioural, ventilatory and cardiac aspects of the reaction are the same in vertebrates as they are in invertebrates, vertebrates are possibly also using it to increase attention. However, until we understand the underlying metabolic and behavioural functions of the alternate response in different animals, we will be unable to make any universal statements about it (Cuadras, 1981). By looking at the entirety of the response in many different animals using non-invasive techniques that allow good temporal resolution, it will become clear whether this is an ancestral reaction or one that has evolved separately several times.

3.7. Acknowledgements

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Chapter 4: Squid egg mops provide sensory cues for increased agonistic behaviour between male squid

4.1. Linking information for this chapter

In chapter 3, I investigated how a sudden stimulus can induce changes in the ventilation rate, heart rate and behaviour of cuttlefish, possibly preparing them for flight. In this chapter, I investigate the stimuli that induce fighting in squid. Unlike the stimuli that induce the Deimatic Display (pre-flight behaviour), the stimuli that induce fighting in squid are less well understood. This chapter has been published in the journal *Animal Behaviour*, volume 66, pages 49-58. It was co-authored with Roger Hanlon and Shelley Adamo. For this chapter, I helped design the experimental protocol; I designed the experimental set-up, performed the experiments, developed and performed the analysis and wrote the manuscript.

4.2. Summary

Males of many species compete for access to females. In order to avoid performing potentially costly agonistic behaviour for their entire adult lives, many group-living males use environmental cues to limit agonistic behaviour to times when it will be of most benefit. Long-finned squid, *Loligo pealeii*, live less than a year and aggregate in mixed- and single-sex schools. Adults participate in several spawning events, then die. During spawning events, males actively compete for females. Winning males pair with females, which subsequently lay eggs in communal sites on the ocean floor ("egg mops"). To determine whether males use sensory cues provided by egg mops to regulate agonistic

behaviour, we conducted four laboratory experiments. We measured the agonistic responses of pairs of adult males before, during and after exposure to conspecific egg mops. In three experiments, egg mops were manipulated to provide differing sensory stimuli (tactile, water-borne, visual). The addition of conspecific egg mops to tanks of paired male squid dramatically increased agonistic behaviour above control levels within minutes. Male squid were first attracted to the egg mops visually, but contact with the capsules was necessary to increase agonistic behaviour. After initial contact, agonistic behaviour was almost continuous as long as egg mops remained present, even when squid touched the egg mops infrequently. Visual stimuli seemed important in maintaining elevated agonistic behaviour between egg mop touches. When egg mops were removed from the tank, measured agonistic behaviour declined within minutes. When egg mops were added to the tank while covered by an opaque and porous cover that allowed water-borne stimuli to circulate into the tank, squid did not approach the covered egg mop or show increased agonistic behaviour. This result suggests that water-borne stimuli are not sufficient to increase agonistic behaviour. It is unusual for male agonistic behaviour in any species to be increased by contact with fertilized eggs. In this species, however, egg capsules might signal that sexually mature, receptive females are about to lay eggs. Indirect evidence suggests that mating with a female immediately before she lays eggs increases male paternity. If this prediction is true, the presence of egg mops may indicate the optimal time for male squid to establish mating precedence through agonistic bouts.

4.3. Introduction

Males commonly compete for receptive, fertile females, the cost being repaid by increased reproductive success (Davies, 1991; Alcock, 1993; Sutherland, 1996; Birkhead

and Parker, 1997). Male-male agonistic behaviour is costly to its participants in several ways. Direct costs of fighting include depletion of accumulated energy stores (Marler and Moore, 1991) and risk of injury and death (Enquist and Leimar, 1990). Indirect costs include increased predation (Lima and Dill, 1990) and decreased time spent foraging (Isvaran and Jhala, 2000).

In several animals, male agonistic behaviour is initiated by exposure to a male conspecific (e.g. cuttlefish, Adamo and Hanlon, 1996). However, animals that live in groups year-round are surrounded by both conspecific males and females at all times. In these animals, selection appears to favour male reproductive strategies that avoid continuous fighting (Davies, 1991). The exact strategy differs from species to species, but males tend to use cues that predict when females are fertile, or are about to become fertile, to target agonistic competition to times when it will have the biggest reproductive benefit. For example, in the savanna baboon, *Papio cynocephalus anubis*, males exhibit increased aggressive competition on days that an oestrus female is most likely to conceive (Bercovitch, 1989). In other words, males use cues from the female to target agonistic behaviour to the short period when they are most likely to sire offspring. In other species, males use seasonal cues to predict increased female fertility and to regulate agonistic behaviour. For example, male Atlantic cod, *Gadus morhua*, show intrasexual agonistic behaviour only during spawning (Hutchings et al., 1999). Spawning seems to be controlled by water temperature, and so both spawning and intrasexual competition are limited to certain times of the year (Hutchings and Myers, 1994).

Agonistic behaviour frequently occurs between male squid in the context of mating behaviour (Hanlon and Messenger, 1996), and has been reported in mating aggregations of *Loligo pealeii*, the long-finned squid of the northwest Atlantic (Arnold,

1962; Hanlon, 1996). Most large males compete directly with each other to win temporary consortships with females (Arnold, 1962; Hanlon, 1996). Only a consort male mates with his female in the "male parallel" position (Hanlon et al., 1997) by placing his spermatophore near the opening of her oviduct (Drew, 1911). In the wild, consorts subsequently guard their mates for several hours (R. T. Hanlon, personal observation). Smaller males, as well as large males that lose competitions with other large males, use "sneaker" tactics. Sneaker tactics of *L. pealeii* involve mating fleetingly in the "head-to-head" position with already paired females and placing their spermatophores in the seminal receptacle of the female, which is far from the oviduct; there is no subsequent mate guarding (Hanlon et al., 1997). All males are able to perform both consort and sneaker matings (Hanlon et al., 1997). Because *L. pealeii* lives less than a year (Brodziak and Macy, 1996), the male's lifetime reproductive success is related to the number of fertilizations that he achieves during one reproductive season.

As in other group-living animals, the regulation of *L. pealeii* agonistic behaviour is an interesting problem. *Loligo pealeii* aggregate in both mixed-sex and single-sex schools. If adult male squid fought every conspecific male, or whenever fertile females were present, most of their day would be spent fighting. Field observations suggest that males do not fight continuously, even during the breeding season. Therefore male squid do not rely solely on the presence of other males, the presence of females or on seasonal cues to regulate agonistic behaviour. How do male *L. pealeii* regulate their agonistic behaviour?

The natural history of *L. pealeii* suggests an answer. *Loligo pealeii* migrates onshore in spring and spawns throughout the summer in shallow, near-shore sandy habitats. Like other squid, this species does not provide parental care to the eggs or to the

hatchlings (Hanlon and Messenger, 1996). Between 100 and 300 eggs are encapsulated in a gelatinous capsule, or "finger" (ca. 20 cm long), that is attached to the substrate (Maxwell and Hanlon, 2000). Observations in the field and in the laboratory indicate that female squid prefer to lay their eggs with those of other females, creating egg mops (Stevenson, 1934; Arnold, 1962; Griswold and Prezioso, 1981). Egg mops can become very large with hundreds of capsules or "fingers". Observations also suggest that only females that are laying egg capsules and mature males approach the egg mops (Arnold, 1962; Griswold and Prezioso, 1981).

The eggs are fertilized not during mating, but as they are deposited. Females arrive at the onshore spawning grounds having already mated and with spermatophores stored in their seminal receptacle. Extensive field video (40 hrs over three seasons; unpublished data) indicates that females mate with multiple males once onshore, usually in the presence of egg mops (Hanlon, 1996; Hanlon and Messenger, 1996; Maxwell and Hanlon, 2000). Actively mating *L. pealeii* are found close to the egg mop, and squid that are not mating are found many meters away (Hanlon et al., 1997).

Stevenson (1934), Arnold (1962), and Hanlon (1996) have all suggested that egg mops help to regulate male agonistic behaviour in *L. pealeii*. We reported initial findings that squid egg mops increase the elevated agonistic behaviour Forward lunge/grab, between mature male *L. pealeii* (King et al., 1999). In this paper, we report the effect of egg mops on five male agonistic behaviours. We also report on the sensory cues mediating this effect.

4.4. General Methods

4.4.1. Collection and Housing of Squid

Between June and August 1999, adult long-finned squid were collected during trawls in the North Atlantic east of Woods Hole, Massachusetts, U.S.A. Once in the laboratory, males and females were kept together in large, oval holding tanks (360 cm long x 240 cm wide x 90 cm deep). The holding tanks were exposed to an ambient light cycle. *Loligo pealeii* are typically found at water temperatures of less than 23°C (Summers, 1983), and data for this study were taken from trials performed at water temperatures between 14.5 and 22.5°C (56 trials, 112 squid).

4.4.2. General Experimental Design

The afternoon before a trial, we selected male squid, 16-21 cm in mantle length, from the mixed-sex holding tanks. Only squid with minimal dermal abrasions, undamaged fins and an intact pen were selected. No squid was used more than once.

We ran each trial with two squid whose mantle lengths differed by less than 1 cm. One squid of the size-matched pair was isolated in a circular tank (3 m in diameter x 0.5 m deep) and was designated the "constant squid". The other squid was isolated in a rectangular tank (132 cm long x 76 cm wide x 43 cm deep) and was designated the "transferee". All isolated squid were fed small fish (*Fundulus* spp.) in the evening. The next morning, the transferee squid was moved to the circular tank at the beginning of the trial. There were two circular tanks and two rectangular tanks allowing us to run two separate trials each day, one after the other, each with different squid. We used 112 squid

in the 56 trials described below. N refers to the number of pairs of squid. We released the animals in the ocean within hours of testing.

4.4.3. Scoring and Analyzing Agonistic Behaviour

Cephalopods, including squid, use rapidly changing, neurally controlled body patterns to signal to each other during successive stages of agonistic conflict (Hanlon and Messenger, 1996). Many signals are conspicuous, making escalation easy to identify (e.g. cuttlefish, Adamo and Hanlon, 1996).

We monitored five agonistic behaviours during trials: White flashing, Accentuated testis, Fin beating, Chase and Forward lunge/grab (Table 4.1.). These behaviours were chosen because they are both representative of escalating male-male agonistic conflict in *L. pealeii*, and obvious to observers. In order to avoid each other during Chase and Forward lunge/grab, squid jetted backward. Smooth circular test tanks allowed the squid to jet along the circumference of the tank without injuring themselves. Squid rarely contacted each other during Forward lunge/grab. When they did make contact, occasionally skin damage would result. We intervened if it appeared that more than skin damage might result from an encounter (i.e. if biting occurred; 1 of 83 trials).

We divided each trial into 2-min time bins. We recorded the number of times a behaviour occurred during each 2-min bin (occurrence score). If a behaviour lasted for more than 5 s, we also recorded its duration (duration score). Durations were rounded down to the nearest 5-s increment, so estimates of duration were conservative. Occurrence and duration scores were recorded for both squid simultaneously, and for each of the agonistic behaviours. To simplify the data, we combined the occurrence and duration scores for each behaviour into a composite value, the agonistic behaviour score.

Table 4.1. Definitions of the five selected agonistic behaviours and their relationship to previously described behaviours

White flashing	An umbrella term for previously described Clear and White head/arms (Hanlon et al. 1999). It consists of a retraction of the chromatophores on the arms, head and mantle of the squid. This reveals the iridophores beneath, rendering the area white or translucent. Squid can limit this expression to one side of the body, and either to the head and arms, or to the mantle.
Accentuated testis	A retraction of the chromatophores above the very white testis.
Fin beating	Parallel alignment of two squid while they rapidly beat their fins against each other. This behaviour is often accompanied by Clear overlaid with dark Fin spots. Occasionally Ventral mantle stripe, a thin, dark stripe along the medial ventral mantle, is seen. Ventral mantle stripe is often accompanied by a lateral compression of the mantle that increases the ventral-dorsal "height" of the squid.
Chase	One squid moves towards the other while the other moves away.
Forward lunge/grab	Sudden forward swimming towards the other squid. Just before contact with the other squid, the approaching squid throws open its arms to reveal its suckers and beak. The approaching squid usually does not contact the other squid, but contact and wrestling occasionally occur. White flashing usually accompanies this behaviour. Also called Open Arm Charge (King et al. 1999).

To standardize units, the occurrence score was multiplied by 1s and added to the duration score. The agonistic behaviour score is therefore an approximation of the seconds spent performing a given behaviour. Forward lunge-grab consistently lasted less than 5 s, so its agonistic behaviour score was based entirely on its occurrence score.

We did not score behaviour during the 2-min breaks used to add or remove stimuli from the tanks.

This study focused on the effect of egg mops on the total amount of agonistic behaviour observed, and not on the effect of egg mops on individual members of the squid pair. Therefore, the agonistic behaviour scores of both squid were added together for a given time bin. Numbers reflect the total amount of a given agonistic behaviour in the tank over a given time period. We analysed each monitored behaviour separately. Nonparametric statistics were used because results showed floor and ceiling effects.

4.4.4. Construction of Egg Mops

In the wild, females attach one end of each egg capsule to the substrate. Usually several capsules are attached in the same place by several females, creating egg mops. Egg mops in the wild contain 5-300 egg capsules. We made experimental egg mops by attaching 16-21 egg capsules to a weight (an airstone) with an elastic band. This number of capsules is sufficient to increase courting and mating in *L. pealeii* (Hanlon, 1996; Hanlon et al., 1997). We used an airstone as a weight because it provided a readily available, uniform way to sink the experimental egg mop and keep it roughly stationary on the tank floor. Egg capsules were used 2-7 days after being laid.

4.5. Experiment 1: The effect of adding egg mops

The purpose of these trials was to compare levels of male agonistic behaviour observed during exposure to an egg mop to those observed during exposure to an airstone. We used an airstone as a control to insure that changes in male agonistic behaviour could be attributed to the presence of the egg mop and not to the addition of a novel object or to the continuing presence of another male in the tank.

4.5.1. Methods

After moving the transferee squid to the circular tank containing the constant squid, we allowed the squid to interact for 30 min (N=5 pairs). Then, during a 2-min break, we added one egg mop to the centre of the tank. We scored the agonistic behaviour of the squid for 30 min while the egg mop remained in the tank.

To test whether observed effects were due to factors not specific to egg mops, such as novel stimuli or continuing exposure to a male conspecific, we performed experiments identical to those above, except that we added a clean airstone to the centre of the tank instead of an egg mop (N=7). The airstone was identical to that used to make the egg mops, except that it had never contacted egg capsules. We controlled for potential tank and time-of-day effects.

We compared agonistic behaviour observed while the egg mop was present to that observed while the airstone was present using a one-way ranked statistical test (specific test for two independent samples; Meddis, 1984). Trial segments in experiments 2, 3, and 4 lasted only 20 minutes (see experiment 2). To facilitate comparisons between the results of all experiments, agonistic behaviour scores reported for this experiment were

summed over the first 20 min of the 30-min exposure to the egg mop or airstone. However, we describe the entire trial below.

4.5.2. Results

Immediately after the transferee squid was moved to the circular tank, the squid swam towards each other. For about the next 10 min, they exhibited the agonistic behaviours described in Table 4.1. (11 of 12 trials). The initial agonistic conflict most often involved visual signals such as White flashing (9 of 12 trials) and Accentuated testis (8 of 12 trials), and less often physical behaviours typical of escalation such as Fin beating (2 of 12 trials) and Chase (4 of 12 trials). Agonistic behaviour usually decreased within 10 min after the transferee had been introduced (e.g. Fig. 4.3., first 20 min of experiment 2). Once agonistic behaviour declined, squid typically swam forwards and then backwards (described in detail by Stevenson, 1934), showing a resting brown colouration with iridescent dorsal spots ("Clear Body Pattern"; Hanlon et al., 1999; 8 of 12 trials). This behaviour is thought to be typical of squid not involved in social signaling (Hanlon et al., 1999).

Within 6 min of adding egg mops, one of the squid swam towards the egg mop, blew water on it with his siphon and touched it with his arms (4 of 5 trials; fifth trial: touch within 10 minutes). Once an egg mop was present, but before either squid had touched it, the squids' agonistic behaviour remained unchanged (5 of 5 trials). However, after either squid touched the egg mop, agonistic behaviour scores increased over a few minutes (e.g. Fig. 4.3., compare min 19 and min 23; data taken from experiment 2). In two trials, additional to the five reported above, neither squid touched the egg mop during the 30 min that it was in the tank. In these trials, the squid continued to show the same

low levels of agonistic behaviour seen before introduction of the egg mops (data not shown).

The agonistic behaviour observed after a squid touched the egg mop contrasted with the agonistic bout observed when the squid were first put together. This behaviour did not subside and involved the escalated behaviour Chase in all trials. Egg mops also triggered Fin beating, a costly but potentially more accurate way for squid to assess each other.

To insure that this increase in agonistic behaviour was due to stimuli specific to the egg mop, we compared the agonistic behaviour observed once egg mops had been added to the tank to the experiments in which a clean airstone had been added. Neither squid touched the clean airstone in any trial (N=7). Agonistic behaviour scores were significantly higher when squid were exposed to egg mops than when squid were exposed to airstones (two-sample approximation test: White flashing: Z'=1.25, NS; Fin beating: Z'=2.73, P=0.01; Accentuated testis: Z'=2.68, P=0.01; Chase: Z'=2.85, P=0.01; Forward lunge/grab: Z'=2.95, P=0.01; Fig. 4.1.).

In all 36 trials that involved egg mops, one squid always touched the egg mop more often than the other squid. The squid that touched the egg mop more during the trial also launched aggressive attacks on his partner, and his partner always retreated and never attacked. We designated the attacking squid the "winner" squid and the retreating squid "looser" squid. Transferee and constant squid were equally likely to be the winner squid. The transferee was the winner in 15 of the 36 trials (Sign Test: NS).

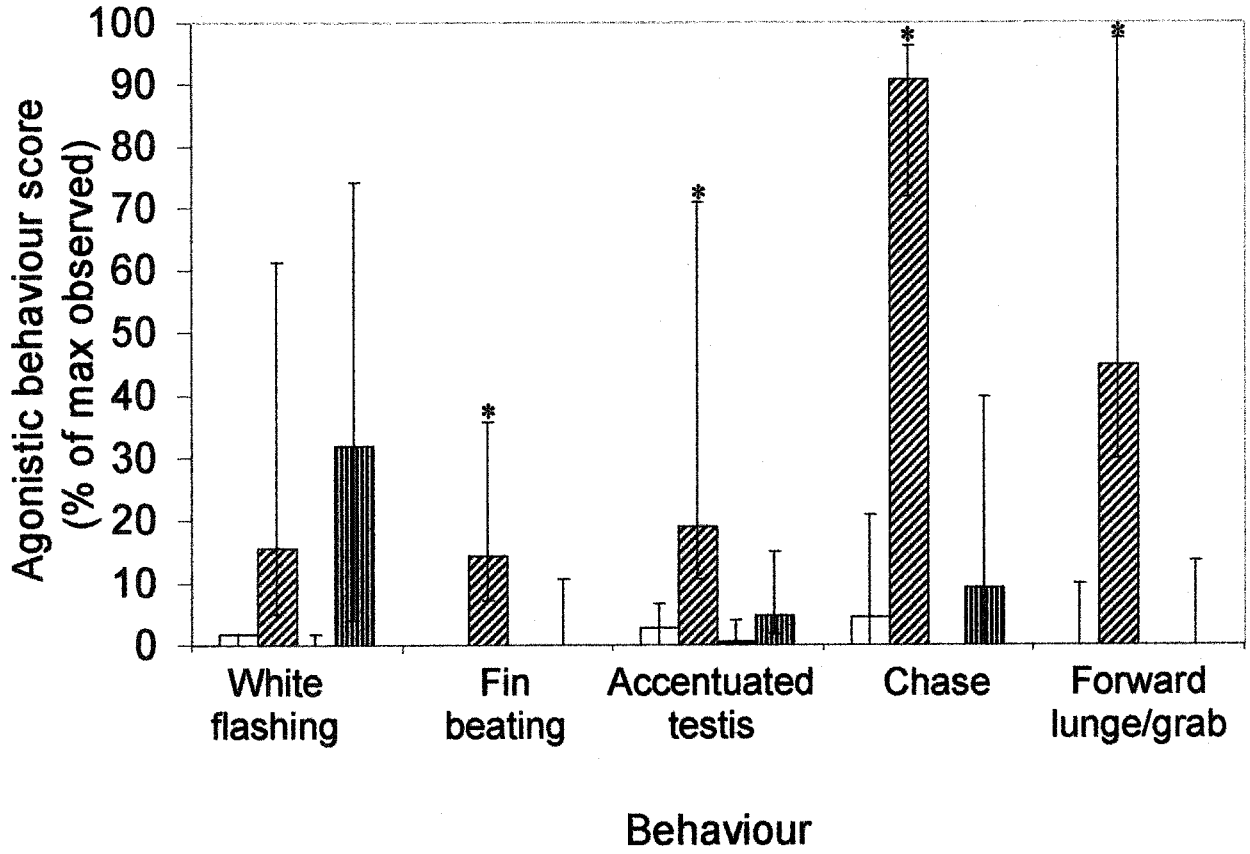

Figure 4.1. Agonistic behaviour score for all measured behaviours during exposure to an airstone (N=7 pairs, □), an egg mop (N=5 pairs, ▨), a covered egg mop (N=7 pairs, ■) or visual stimuli from an egg mop (N=8 pairs, ▥). Each behaviour had a different range of agonistic behaviour scores. Therefore, values were converted to percentages using the highest value observed for a given behaviour (a different maximum was used for each behaviour). * $P<0.01$ compared with the airstone treatment.

4.6. Experiment 2: Duration of egg mop effect

Experiment 2 was performed to determine how long our selected agonistic behaviours remained elevated after egg mops were removed from the tank.

4.6.1. Methods

In experiment 1, when squid were initially introduced and egg mops were absent, the agonistic behaviours that we measured declined almost to zero after 10 min. Within 20 min after the addition of egg mops, agonistic behaviour between the squid appeared to plateau. Therefore, we shortened the pre-egg mop and egg mop-exposure segments of this experiment to 20 minutes each.

We moved the transferee to the circular tank, and for 20 min, scored the agonistic behaviour of the squid (N=16 pairs). We then added three egg mops during a 2-min break. We placed one egg mop in the centre of the tank, and the other two near the walls on opposite sides of the tank. We used three egg mops to insure that squid had optimal opportunity to contact the egg mop. After scoring the agonistic behaviour during egg-mop exposure for 20 min, we removed the three egg mops from half the trials, and replaced them with three airstones (N=8). We scored the agonistic behaviours of the squid for 30 min after egg mop removal.

In the other half of trials (N=8 pairs), egg mops were removed then returned to the tank during a 2-min break to mimic the tank disturbance that occurred when we removed egg mops and replaced them with airstones. Egg mops were then left in the tank for 30 min. These trials were used as a control to insure that agonistic behaviour did not spontaneously decrease even when egg mops remained in the tank. These squid pairs

were exposed to the egg mops for a total of 50 min. We controlled for tank and time-of-day effects.

To determine how quickly the agonistic behaviour induced by the egg mop subsided once egg mops were removed, we divided the last 30 min of the trials into three 10-min segments. Agonistic behaviour scores were summed separately for Accentuated testis, Chase and Forward lunge/grab over each of these 10-min segments. White flashing and Fin beating were not tested because they were seldom seen during this period. We compared the agonistic behaviour score for each behaviour summed over each 10-minute segment from trials in which egg mops were removed, with its counterpart in trials where egg mops were returned to the tank. We used a one-way ranked test (specific test for two independent samples, Meddis 1984).

4.6.2. Results

The agonistic behaviour scores for Accentuated testis, Chase and Forward lunge/grab decreased significantly over the first 10 min after egg mops were removed from the tank (two-sample approximation test: Accentuated testis: Z'=2.85, P<0.01; Chase: Z'=2.63. P<0.01; Forward lunge/grab: Z'=1.64, P<0.05; N=16 pairs; Fig. 4.2.). In tanks from which egg mops were removed, agonistic behaviour scores remained significantly lower for all three behaviours over the rest of the trial than when egg mops remained in the tank (Fig. 4.2.). Forward lunge/grab, the most aggressive behaviour measured, declined to zero by 30 min after the removal of egg mops (Fig. 4.3.). The other two behaviours did not fall to zero 30 minutes after egg mop removal, but were at significantly lower levels than during egg mop exposure (Fig. 4.3.).

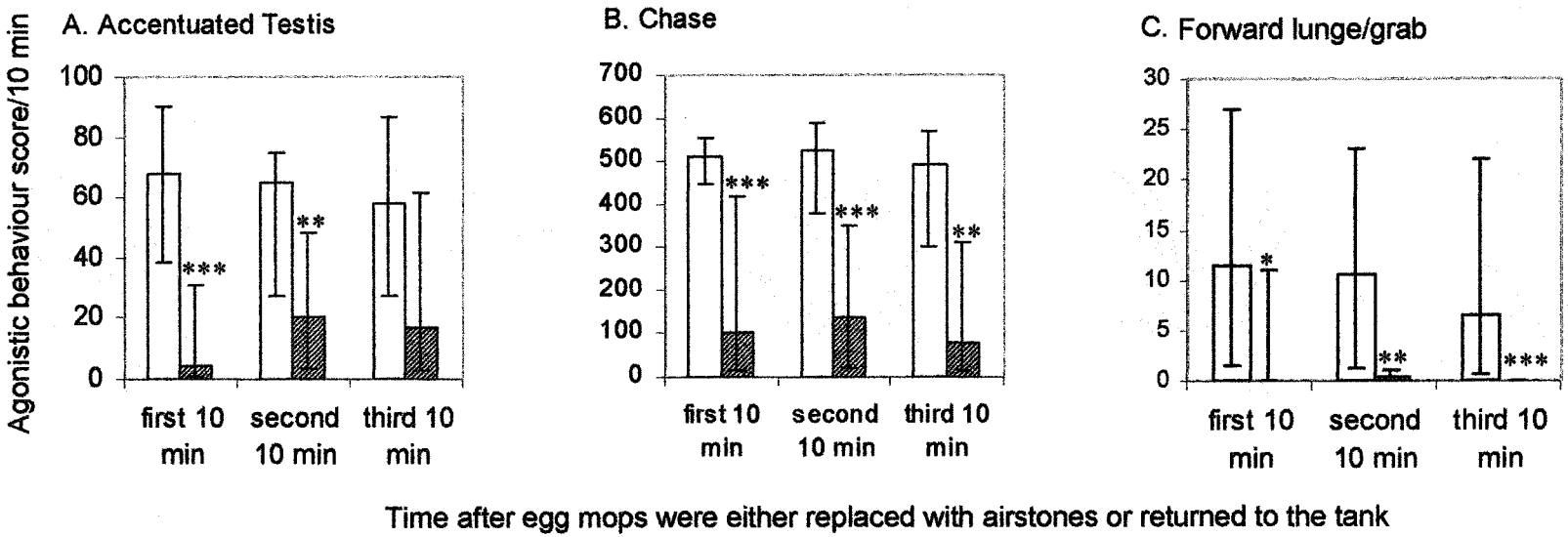

Figure 4.2. Agonistic behaviour score for A. Accentuated testis, B. Chase and C. Forward lunge/grab after egg mops were either removed briefly, then returned to the tank (N=8 pairs; □), or replaced with airstones (N=8 pairs; ▨). Agonistic behaviour was elevated before egg mop manipulation because squid had already been exposed to egg mops for 20 min. Once egg mops were replaced with airstones, agonistic behaviour was lower over three consecutive 10-min segments than if egg mops remained in the tank. Specific test for two independent samples: * P=0.05; ** P<0.025; *** P<0.01.

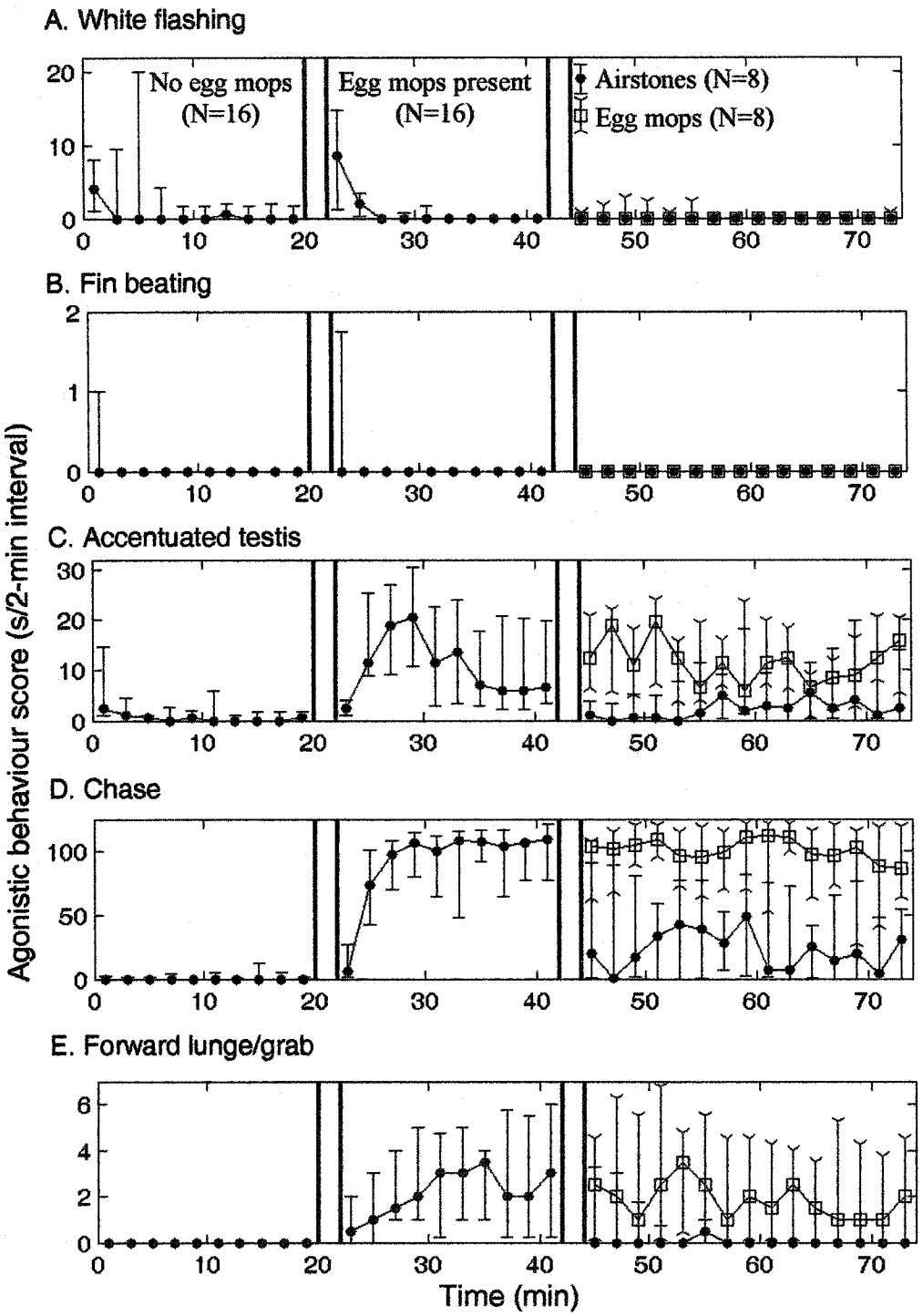

Figure 4.3. Agonistic behaviour scores for each of the five monitored agonistic behaviours for each 2-min interval in experiment 2. Twenty minutes after the transferee was added, the squid were exposed to three egg mops for 20 min (N=16 pairs). In eight trials, the egg mops were removed and three airstones added. In the other eight trials, the egg mops were removed briefly, then replaced. Plotted values are medians ± first and third quartiles.

Results from experiment 1 suggested that touching egg mops initiated elevated agonistic behaviour between male squid. Results from experiment 2 suggested that the presence of egg mops was necessary to maintain agonistic behaviour once it had been initiated. However, it was not clear whether touching the egg mop was required to maintain agonistic behaviour between squid once it had been initiated. Squid touched the eggs a median number of two times every 10 min (first quartile = 1.4 times, third quartile = 3.6 times, N=16 pairs), that is, there were often several minutes between touches. In trials in which egg mops were removed and then immediately replaced in the tank, some squid did not touch the egg mops for at least 4 min after they were replaced (N=5). Note that in these trials, males were already engaging in elevated agonistic behaviour from being exposed to egg mops in the first 20-min segment. Although they did not touch the egg mops for 4 min after they were returned to the tank, agonistic behaviour remained elevated. This contrasts with results obtained from parallel trails, in which egg mops were removed and replaced with airstones. In these trials, squid were not only prevented from touching the egg mops (because egg mops had been removed), but they were also deprived of any other stimuli the egg mops might provide. There was significantly less Accentuated testis 4 min after egg mops were removed than in experiments in which egg mops were present but not touched (specific two-sample approximation test: Z'=2.64, N=13, $P<0.05$). Despite a similar pattern, there was no significant difference in Chase or Forward lung/grab between the groups (Fig. 4.4.). White flashing and Fin beating were not tested because they were seldom observed during this period. These results suggest that a nontactile stimulus maintains agonistic behaviour over several minutes when egg mops are present. Potential water-borne chemical stimuli released by the egg mop would

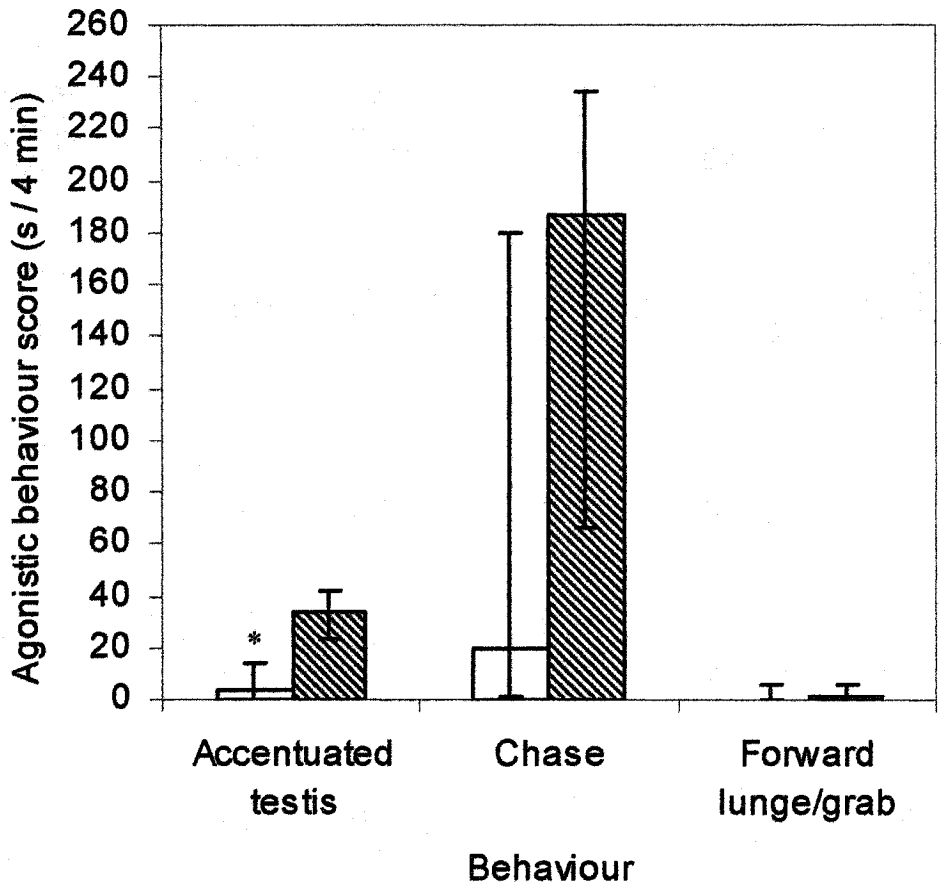

Figure 4.4. Agonistic behaviour scores for Accentuated testis, Chase and Forward lunge/grab over the 4 min after egg mops had been removed and then immediately replaced (N=5 pairs; ▨), or removed and replaced with airstones (N=8 pairs; □). Only trials in which squid did not touch the egg mops during the four minutes were included. * Significantly less than when egg mops were present (specific two-sample approximate test: Z'=2.64, N=13, P<0.05).

not have been able to clear the tank quickly enough to coincide with the decrease in agonistic behaviour seen after egg mop removal. Visual cues, however, may be important in maintaining agonistic behaviour between egg mop touches.

4.7. Experiment 3: Is the egg mop sensed by water-borne chemical stimuli?

Experiment 2 showed that squid pairs not only increased agonistic behaviour when egg mops were present, but also decreased agonsitic behaviour when egg mops were removed. How did squid find the egg mops, and which senses were important for increasing agonistic behaviour? *Aplysia*, another mollusc, finds and aggregates around egg masses by following water-borne chemicals released by egg cordons (Painter et al., 1991). In experiment 3, we investigated the role of potential water-borne chemical stimuli released by the egg mop in attracting squid to the egg mop and in initiating agonistic behaviour.

4.7.1. Methods

An egg mop was added to the tank under an opaque, perforated cover, 20 min after the transferee was moved to the circular tank (N=16 pairs). The cover was approximately cylindrical (10 cm in diameter x 14 cm tall). There were two holes, 0.6 cm in diameter, in the top of the cover. There were 90 more holes in the side of the cover, which were approximately 0.3 cm in diameter. The cover obscured the egg mop visually, both as it was being added to the tank and while it was in the tank. It did, however, allow possible water-borne chemical substances to circulate in the tank; in separate observations, a bolus of phenol red dye injected under the cover flowed out freely. Water

circulated in the tank during all trials. The covered egg mop was in the tank for 20 min. We scored the agonistic behaviour of the squid during this time.

Occasionally in previous trials, some pairs of squid did not notice or touch the egg mops, and there was no subsequent increase in agonistic behaviour. It was not clear why squid did not touch the egg mops in those trials, but we suspected that some squid were not motivated to engage in agonistic behaviour. If a pair of squid did not increase their agonistic behaviour in the presence of a covered egg mop, we could not determine whether the squid were simply unmotivated to respond to egg mops, or whether water-borne chemicals were unimportant in increasing agonistic behaviour. Therefore, we tested the squid at the end of all trials by exposing the egg mop. We removed the cover during a 2-min break, and left the egg mop exposed in the tank for 20 min. We scored agonistic behaviour during that time. Only trials in which the squid touched the egg mop and increased agonistic behaviour once the cover was removed were used in the analysis (touched in 7 of 16 trials), because we were certain that these squid were responding normally to egg mops.

We summed agonistic behaviour scores recorded while the covered egg mop was in the tank for 20 min and compared this with scores recorded during airstone exposure in experiment 1, using a two-way ranked test (nonspecific test for two independent samples; Meddis, 1984) to control for disturbance effects.

4.7.2. Results

Squid often briefly oriented towards the opaque, perforated egg mop cover as it was lowered into the tank, but they never approached or touched it. Agonistic behaviour was not significantly different from control values while the covered egg mop was in the

tank, both for pairs that subsequently touched the exposed egg mop (covered egg mop versus airstone; Fig. 4.1.), and for those that did not (data not shown). Once the egg mop was exposed, there was no increase in agonistic behaviour unless the squid touched it (touch in 7 of 16 trials, agonistic behaviour increased in all 7 trials). Squid that did not touch the egg mop, also did not notice or approach it. They continued exhibiting the low levels of agonistic behaviour that we observed before and during exposure to the covered egg mop (9 of 9 trials). At the conclusion of five trials in which the squid did not touch the egg mop, we moved the egg mop around the tank with a stick. This induced the previously unresponsive squid to touch the egg mop. In all cases, agonistic behaviour increased (data not shown). This result supports our conclusion that touch is necessary and sufficient to increase agonistic behaviour above control levels.

Why so many squid did not touch the egg mop in this experiment is not clear. It is possible that motionless egg mops do not attract squid. Simply removing the opaque cover from the egg mop may not have caused enough movement of the egg mop to attract the attention of all squid pairs, a problem not encountered when placing egg mops in the tank from above as in other experiments. When egg mops were moved around the tank, previously non-responsive squid then touched the egg mops and increased their agonistic behaviour. In nature, it is possible that waves move the egg capsules creating a necessary part of the attractive visual cue for squid. This hypothesis, however, remains to be tested.

4.8. Experiment 4: Do squid use vision to detect egg mops?

Arnold (1962) suggested that *L. pealeii* uses visual stimuli alone to detect egg mops and to increase agonistic behaviour. In this experiment, we tested the effect of

visual stimuli, combined with any potential diffusible chemical stimuli, on increasing male squid agonistic behaviour.

4.8.1. Methods

The night before a trial, we placed an open-topped glass box (21 cm high x 15 cm wide x 30 cm long) in the circular tank (N=12). The next morning, after allowing the squid to interact for 20 min, we put an egg mop into the glass box during a 2-min break. The agonistic behaviours of the squid were scored for 20 min while the egg mop was in the glass box. Despite its being open at the top, squid never swam into the box to touch the egg mop. Then, during a 2-min break, we placed the egg mop outside the glass box. We scored the agonistic behaviour for another 20 min to test whether squid that were unresponsive to the egg mop in the glass box were generally unresponsive to egg mops. Using the same rationale as in experiment 3, we report results only for trials in which squid touched the egg mop once it was placed outside the glass box (8 of 12 trials).

We summed agonistic behaviour scores recorded while the egg mop was in the glass box (20-min period) and compared these to scores recorded during airstone exposure in experiment 1, using a one-way ranked test (specific test for two independent samples, Meddis 1984), to compensate for disturbance effects.

4.8.2. Results

Once an egg mop was placed in the glass box, squid frequently approached and attempted to touch the egg mop through the glass (for 20 min: median, first quartile and third quartile, respectively: 10.0, 4.75, 18.5, N=8). Thus, the sight of an egg mop seemed to attract the squid. During the 20 min when squid could see but not touch the egg mops,

agonistic behaviour scores were not significantly higher than when airstones were added (egg mop in glass box, N=8 pairs, versus airstone, N=7 pairs; Fig. 4.1.). Agonistic behaviour did increase significantly after squid touched the egg mop, once it was placed in front of the glass box (touch occurred in 8 of 12 trials, data not shown).

4.9. Discussion

4.9.1. Sensory stimuli that affect agonistic behaviour

The introduction of egg mops increased agonistic behaviour over control levels between paired male long-finned squid. Visual cues attracted squid to the egg mop, but contact with the egg mop was necessary before agonistic behaviour increased over control levels. Agonistic behaviour specific to egg mop exposure remained elevated as long as egg mops were present, even though females were absent, and decreased when egg mops were removed. Squid touched the egg mop several times during a trial, but there were often several minutes between touches. Visual stimuli, in addition to initially attracting squid to the egg mop, are probably important in maintaining agonistic behaviour between touches. Water-borne chemicals released by the egg mop do not appear to be attractive and are not sufficient to increase agonistic behaviour over control levels.

The importance of touching the egg mops might have evolved because *L. pealeii* shares its range with other squid such as *L. plei* and *Lolliguncula brevis* (Roper et al., 1984). All have a similar egg capsule morphology. Although the squid might find egg mops by sight, contact with species-specific chemicals embedded in the capsules of the eggs might inform the male squid whether the egg mop was laid by a conspecific, and therefore whether it is an appropriate time and place to increase agonistic behaviour. The

effect of egg mops laid by other species on *L. pealeii*'s agonistic behaviour should be investigated.

Egg mops may not be the only modulators of agonistic behaviour in *L. pealeii*. The power of a conspecific adult female to increase the intensity of male agonistic behaviour has been shown in *L. plei* (DiMarco and Hanlon, 1997), a closely related and sympatric species of *L. pealeii*. It is possible that, as in other animals (Crews and Moore, 1986), multiple cues (e.g. from both females and egg mops) regulate specific aspects of agonistic behaviour in squid.

4.9.2. Association between increased male agonistic behaviour and egg mops

Increased agonistic behaviour when offspring are present is commonly seen in vertebrates and invertebrates that exhibit some degree of parental care (Montgomerie and Weatherhead, 1988; Maestripieri, 1992; Figler et al., 1997; see Archer, 1988 for review). Considering that, in squid, neither sex provides parental care to the eggs or to the hatchlings (Hanlon and Messenger, 1996), we do not believe that the increase in male agonistic behaviour seen in this study is related to offspring defense. In other decapod cephalopods, reported male-male competition of this kind is for mates and not for other resources such as food or shelter (Hanlon and Messenger, 1996). It is not clear from this study whether males are competing directly (winners get females) or indirectly (winners get resources that make them more attractive to females) for mates. However, it is clear from field observations that winning males become the consorts of females (Hanlon et al., 1997), so the agonistic contest seen in this study is probably ultimately for access to females. In this study, males appeared to use fertilized eggs as a cue to escalate male-

male agonistic competition for females. We know of no other group-living animal that uses fertilized eggs to trigger male-male competition.

Other group-living animals, such as red deer, *Cervus elaphus*, use seasonal cues to trigger a short but intense breeding season in which males spend most of their time performing agonistic behaviour (Lincoln et al., 1972). Although squid are also seasonal breeders (breed only during certain times each year), reproductive behaviour is possible during much of their short lifespan (Maxwell and Hanlon, 2000). Squid agonistic behaviour may be too costly to maintain over such a long breeding season; instead nonseasonal cues may allow agonistic behaviour to be targeted to certain periods within the season. In some animals, cues from the female are necessary for increased agonistic behaviour (e.g. bowl and doily spiders, *Frontinella pyramitela*: Austad, 1983; white-crowned sparrows, *Zonotrichia leucophrys*: Moore, 1984), but this is not true in *L. pealeii*. The presence of receptive females may be continuous in this group-living animal, and cannot alone be used to target agonistic behaviour to optimal times. We hypothesize that increasing agonistic behaviour in the presence of fertilized eggs allows males to target their agonistic behaviour to times and places when mating will result in the most offspring.

Some indirect evidence supports this hypothesis. In birds and insects in which no physical barrier to the female's reproductive tract is placed by previously mating males (as is the case in squid), last-male sperm precedence is commonly seen (Birkhead and Parker, 1997). Females of the genus *Loligo* mate with several males and multiple paternity is present within eggs of one capsule (Shaw and Boyle, 1997; Buresch et al., 2001). One male is more successful than the others at fertilizing the eggs in a given capsule. In *L. pealeii*, one male fertilizes more than half of the eggs in any egg capsule

(Buresch et al., 2001). Winner males mate in the "male parallel" position and deposit their spermatophores near the opening of the female's oviduct, rather than in the suboral seminal receptacle, as is done in "sneaker" and offshore matings. From in vitro observations of spermatophores, Drew (1911) estimated that spermatophores continue to release sperm for as long as 2 days after they are deposited. Because winning males will often guard their mates for a few hours while continuing to mate with them (Hanlon et al., 1999), it is possible that the female's mantle could temporarily be filled predominantly with the consort (i.e. winner) male's sperm. If eggs were laid during this time, winner male sperm would contact the egg capsule before the sperm stored in the seminal receptacle near the mouth, perhaps conferring an advantage to winner male's sperm. Because females near egg mops are likely to lay eggs soon, egg mops could signal when it is most advantageous to compete to be a winner male and subsequently mate, placing spermatophores advantageously near the end of the oviduct. Although more testing is required to show that these mechanisms enable winning males to fertilize an increased number of eggs, it is likely that the costs associated with increased agonistic behaviour near egg mops are repaid by increased paternity.

Loligo pealeii may not be alone amongst near-shore squid species in using egg mops to cue sexual competition. Male investigation of egg mops has been observed in both *L. vulgaris* (Tardent, 1962) and *L. opalescens* (R. T. Hanlon, personal observation) during sexual behaviour. The ubiquity across squid species, and perhaps across other classes of molluscs, of this unusual cue for increasing male-male sexual competition remains to be investigated.

4.10. Acknowledgments

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Chapter 5: Conclusion

5.1. Similarities and differences between coleoids and vertebrates

Coleoid cephalopods and vertebrates face similar challenges because of their active lifestyles and complex behaviour. Many of the solutions to these challenges are functionally similar (Packard, 1972). However, the details of the solutions differ between the two groups and are shaped by their respective ancestral *Bauplans* (Wells, 1983; O'Dor and Webber, 1986). In the thesis, I have investigated three challenges that cephalopods have solved in ways that differ in detail from vertebrate solutions: 1. how to power venous return, 2. how to respond to sudden stimuli, and 3. how to regulate male agonistic behaviour.

5.1.1. Venous return

In chapter 2, I presented evidence that all large veins, including the anterior vena cava, actively contracted in cuttlefish. This is likely also true of octopods and squid. In octopods, the large veins contract in dissected animals (Smith and Boyle, 1983) and anesthetized octopods with their mantles turned inside out (Wells and Smith, 1987). Furthermore, the *in vivo* pressure traces from the large veins of the octopus *Enteroctopus dofleini* Wülker (Johansen and Martin, 1962) corroborate my results (see section 5.2.1.). In squid, it would not be surprising to find that the large veins contract, given that the anatomy and innervation of their large veins, branchial hearts and systemic hearts are almost identical to those of cuttlefish (see chapter 1, section 1.1.2.). Furthermore, *in vivo*

pressure traces from squid's large veins are similar to those of the octopus (Bourne, 1982) and corroborate my results.

Previous studies have shown that, in addition to the large veins, the smaller veins of the arms and head of *E. dofleini* also actively contract (Smith, 1962). It seems that all the veins actively contract in octopods, and probably also in other coleoid cephalopods. It is not clear why coleoid cephalopods power venous return by using contractile veins instead of using the mammalian system of one-way valves and muscular compression of the veins (Withers, 1992). It may be because the large veins span areas where there are no external muscles to compress them (i.e. the mantle cavity and the visceral mass). Peristalsis may therefore be the only method to move blood through the large veins.

The coleoid circulatory system appears superficially to be avian or even mammalian because of the separated systemic and branchial circulations. However, the extensive use of contractile veins to power venous return makes the circulatory system of coleoids distinctly different from that of birds and mammals, where contractile veins are a rarity (Smith, 1962; Schipp, 1994).

5.1.2. Responses to sudden stimuli

When exposed to sudden visual stimuli, cuttlefish (chapter 3), octopods (Wells, 1980; Wells et al., 1987) and many other animals (Table 3.1.) respond similarly: heart rate and ventilation rate decrease and the animal exhibits behavioural freezing. However, while most vertebrates experience increased cardiac function during locomotion, jetting octopods experience interrupted cardiac function. The interrupted cardiac function in jetting octopods resembles that seen after exposure to sudden stimuli (Wells et al., 1987).

Interrupted cardiac function may also occur in jetting cuttlefish, although this has never been tested. The mechanisms underlying decreased heart rate in cuttlefish and octopods after sudden stimuli could be shaped by their unusual mode of escape locomotion (jetting), and to particularities of their circulatory system (see section 5.2.2.), rather than to a universally adaptive process that could apply to any animal. It would be interesting to test the squid's reaction to sudden stimuli. Squid do not show interrupted cardiac function during jetting, and may not suffer from the same circulatory constraints that cuttlefish and octopods do.

5.1.3. Regulation of male agonistic behaviour

Male-male competition for females is costly, and we expect it to be regulated by external cues in group-living animals (Davies, 1991). The squid, *Loligo pealeii*, lives in groups and the males compete for females during the breeding season. In chapter 4, I described how male *L. pealeii* used the tactile and visual cues provided by egg mops to regulate agonistic behaviour. Other shallow-water squids such as *L. vulgaris* possibly also use egg mops to regulate agonistic behaviour (Tardent, 1962). The use of fertilized eggs to regulate male agonistic behaviour is not seen outside the squids. However, male-male competition is not the only behaviour to be seen more frequently near egg mops than elsewhere; female egg laying and squid mating are also seen more frequently near egg mops (Arnold, 1962; Griswold and Prezioso, 1981; Hanlon et al., 1997). Egg mops could be a cue for a whole suite of behaviours associated with reproduction. In other molluscs such as *Aplysia*, general reproductive behaviour is facilitated by chemicals released by egg cordons containing fertilized eggs (Painter et al., 2003). That squid use

the unusual cue of fertilized eggs to regulate male-male agonistic behaviour could be a molluscan trait.

Studies of the physiological changes that accompany fighting in cephalopods have not been undertaken. However, now that we have a reliable cue to elicit male fighting behaviour, we can examine cardiovascular and ventilatory changes during fighting, and possibly assess its physiological cost. It would also be interesting to examine how cardiovascular changes during fighting in squid compare to the cardiovascular changes in cuttlefish after startling stimuli.

5.2. New insights into the connection between circulation, ventilation and locomotion

The implications of this thesis extend beyond providing a contrast to the vertebrates; the information in chapters 2 and 3 can be synthesized into a new model of coleoid circulation. The results in these chapters were limited to cuttlefish that were not swimming or jetting. However, the results can be extrapolated to suggest how coleoids are capable of circulating the large amounts of blood necessary to meet their large oxygen demands during exercise.

It has been attractive to many authors to ascribe some circulatory function to the contractions of the mantle. The coleoid mantle is muscular and capable of creating large pressures in the mantle cavity, especially during jetting. During resting ventilation, maximum pressures in the mantle cavity are around 0.16 kPa in cuttlefish (pers. obs.). During jetting, mantle pressures can rise to over 5.5 kPa in cuttlefish (O'Dor and Webber, 1991), 8 kPa in *Octopus* (Wells et al., 1987) and 6.6 kPa in squid (O'Dor and Webber, 1991). Forces generated by the muscular mantle could help to circulate the large amounts

of blood needed during exercise. Below I describe some of the circulatory roles that have been ascribed to the mantle previously, why they are unlikely to be true, and a new model for the mantle's role in circulation.

5.2.1. Previous theories of the mantle's contribution to venous return

It might seem most efficient for the hearts to contract at the same time as the mantle. This way, the increasing mantle pressures could help the hearts to push blood to the periphery, and the slightly negative pressures created during mantle expansion could help to pull venous blood back towards the hearts. However, a 1:1 ratio of heart to mantle contractions is not usually observed in octopods (Wells, 1978; Smith, 1982), squid (Shadwick et al., 1990), or cuttlefish (Chichery, 1980, chapters 2 and 3), even during jetting. Moreover, many authors report that the ratio of contractions between the heart and the mantle can change over time within the same octopus (Johansen and Martin, 1962; Wells, 1978) or cuttlefish (chapter 2). In octopods (Wells, 1978) and squid (Wells et al., 1988; Shadwick et al., 1990), the heart rate is usually higher than the ventilation rate, whereas in cuttlefish, the situation is reversed (Chichery, 1980, chapter 2). While there appears to be no direct correlation between mantle and heart contractions, mantle pressures could have effects on blood movement in the veins and arteries.

Pressures have been measured in the vena cava cephalica and efferent gill vessel of the octopus *E. dofleini* (Johansen and Martin, 1962) and the squid *L. pealeii* (Bourne, 1982). In these vessels, there are two overlaid pressure pulses; one that is relatively slow and large overlaid with one that is relatively fast and small (Fig. 1.4.). The slow, large pulse is due to ventilatory movements (Fig. 1.4.B.). The results of chapter 2 suggest that

the smaller ones are due to venous contraction (Fig. 1.4.C). Probably due to the large size of the ventilatory pulse, many have suggested that pressures created by the mantle flatten large veins such as the venae cavae and the efferent branchial vessels. However, in order for the veins to flatten, the compression-resistant blood inside them must move into the adjacent vasculature. To move blood between veins within the mantle cavity, mantle contractions must generate pressure differences between those veins. Theoretically, the pressures created by the mantle, while large at times, are applied equally to all veins within the mantle, and therefore would not create the pressure differences required to move blood between vessels. The only exception is the anterior vena cava (Fig. 1.2: AVC.), which is fed by the low pressure vasculature outside the mantle. However even this cannot flatten because blood cannot flow backwards out of the anterior vena cava past the valves guarding its entrances (see discussion of anatomy, section 1.2.2.). So, while the contractions of the mantle create absolute pressure changes in the vessels, these are unlikely to be propulsive because they are unlikely to create pressure differences between the vessels. Misinterpretation of the significance of absolute pressures measured in the large veins have muddied the data interpretation of several authors (Johansen and Martin, 1962; Bourne, 1982; Smith, 1982; Wells and Smith, 1987). Future studies should measure the difference between the pressures of adjacent veins to determine whether intravascular pressures are propulsive.

Aside from this theoretical argument, data from chapters 2 and 3 support the hypothesis that increasing mantle cavity pressure has little to do with venous compression. If pressures within the mantle cavity compressed the veins, we would expect their pulses to be tied to the movements of either the mantle or the collar flaps. Three different observations show that they are not. First, in chapter 2, I presented data

showing that the lateral venae cavae and efferent branchial vessels do not change size in time with mantle movements. Second, further data in chapter 2 shows that the anterior vena cava contracts peristaltically. Peristaltic contractions of the anterior vena cava are inconsistent with the pressures generated by the mantle. Third, contractions of the anterior vena cava of a mating female cuttlefish remained steady and slow during the rapid and vigorous mantle flushing that accompanied the placement of the male's arm in her mantle (Video 5.1.). Because the contractions of the large veins are not tied to the movements of the mantle or the collar flaps, I conclude that the veins are not compressed by the mantle and that pressures in the mantle cavity do not aid venous return.

Not only are contractions of mantle not propulsive in the veins, they could impede venous flow from the head and arms into the anterior vena cava by increasing the pressure in the anterior vena cava and without increasing pressure in the venous spaces of the head and arms (Wells et al., 1987).

5.2.2. Hypothetical circulatory function in cuttlefish

The mantle may still aid circulation, not by the pressures it creates in the mantle cavity, but instead by the pressures it creates within its own tissues. The mantle is composed almost exclusively of two muscle types, radial and circular muscles (Fig. 5.1.). Radial muscles contract to expand the mantle during all types of ventilation. Circular muscle contraction constricts the mantle only during heavy ventilation and jetting. Both sets of muscles are partially antagonized by variously arranged collagen tunics, and, during all but resting ventilation, by each other (Bone et al., 1994).

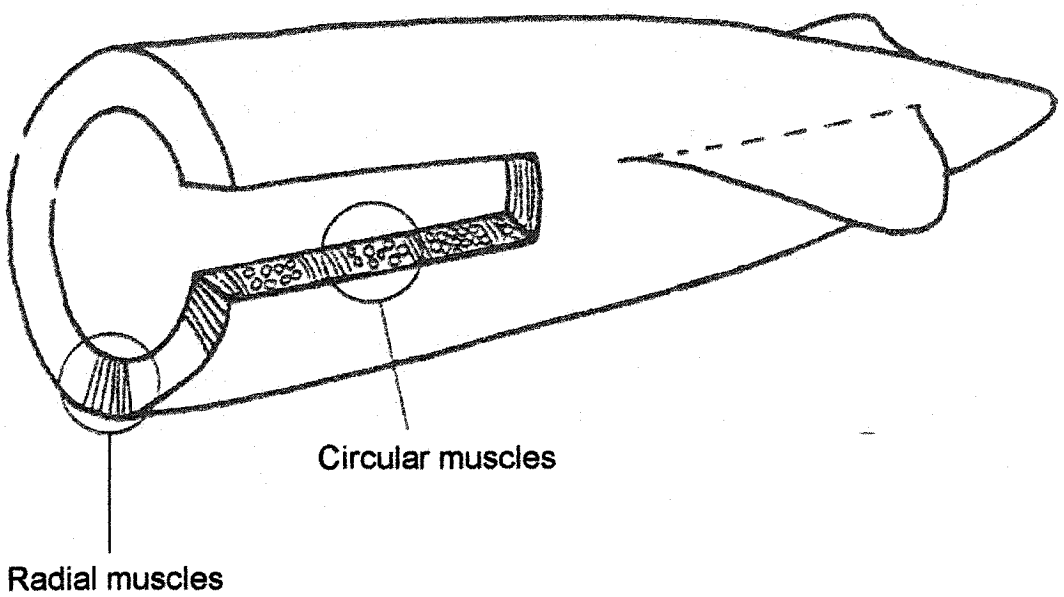

Figure 5.1. The bands of radial and circular muscles in the coleoid mantle. Radial muscles contract to expand the mantle. Circular muscles contract to constrict the mantle. After Shadwick, 1994.

Most capillaries in the mantle are oriented perpendicularly to the radial muscles (Bone et al., 1981) and therefore could be compressed by radial muscle contraction. Conversely, the capillaries are oriented parallel to the circular muscles (Bone et al., 1981), and therefore would not be compressed by circular muscle contraction and in fact, could be expanded by it. Since the radial muscles are always used to expand the mantle, albeit minimally during resting ventilation (Bone et al., 1994), they may always create a bellows-like effect on the mantle capillaries; contracting and forcing blood from the capillaries, relaxing to create a vacuum in the expanding capillaries. This effect would be magnified during jetting when radial muscles contract more vigorously, expelling more blood, and when the circular muscles become active, contributing to radial muscle stretching and possibly vessel dilation. In other words, mantle expansion could aid the flow of blood from the mantle into the veins, while mantle constriction could aid blood flow into and within the mantle. The alternating contractions of the radial and circular muscles could create pumping forces that help to power circulation during exercise.

Unfortunately, almost no direct observations have been made on blood flow in the periphery which, considering the strength and size of the mantle, hinders to our understanding of integrated cardiovascular dynamics (Bourne, 1984). However, empirical data support my hypothesis that mantle muscle contraction influences peripheral circulation. Cuttlefish hearts slow or stop during hyperinflation (chapter 3). The hearts of octopods also slow or stop during jetting, although it was not noted whether this was during hyperinflation or water explusion (Johansen and Martin, 1962; Wells et al., 1987). During hyperinflation, the radial muscles might compress the mantle capillaries, greatly reducing blood flow through the mantle. The cuttlefish or octopus heart might slow or stop at this time to avoid dangerous pressure increases in the head and

viscera, where blood can still flow. The anterior vena cava and ventricle both filled during cardiac slowing or stopping in cuttlefish, even in the absence of the propulsive forces normally generated by the heart (chapter 3). It would seem that venous pressure is maintained during cardiac slowing or stopping, perhaps by the compression of capillaries in the mantle. Lastly, in *Octopus*, pressure in the cephalic aorta remains constant or even increases during cardiac arrest, suggesting increases in peripheral resistance (Johansen and Martin, 1962). Stable or increasing aortic pressures are predicted if radial muscle contraction does collapse mantle capillaries.

Interestingly, the mean resistance of the peripheral vessels remains constant or even decreases during exercise in *Octopus* (Wells et al., 1987) and squid (Shadwick et al., 1990). The contractions of the circular muscles might dilate mantle capillaries, resulting in periods of lowered resistance between the periods of higher resistance associated with radial muscle contraction. The alternating high and low resistance could result in no change in the mean resistance.

While ultimately helping to circulate blood, strong contractions of the radial muscles appear to interfere with cardiac function in cuttlefish (chapter 3) and octopus (Johansen and Martin, 1962; Wells et al., 1987). Squid, however, jet almost constantly, and do not experience cardiac interruptions when jetting. This could be because they regulate blood pressure peripherally with their "peripheral hearts", instead of centrally with their systemic heart. Octopods and cuttlefish do not have peripheral hearts, perhaps because they jet infrequently. Squid, unlike cuttlefish, also seem to rely more on their circular muscles than their radial muscles for mantle shape change (Gosline et al., 1983; Bone et al., 1994). By using their circular muscles, squid may avoid the deleterious effects of radial muscle contraction on circulation.

In cuttlefish, decreasing heart rate does not appear to be as detrimental in the short term as we would expect. Cuttlefish lower their cardiac output and ventilation rate for as much as 20 s, and then swim for many minutes without first increasing these rates above resting. Cuttlefish may not be as close to the edge of oxygen peril as the oxygen content of their venous blood would suggest. They may have other means for oxygen uptake in the tissues. In octopus and squid, a significant amount of oxygen (up to 70% in exercising squid) diffuses directly into the mantle tissues from the water inside and outside the mantle cavity (Wells and Wells, 1983; Wells et al., 1988; Pörtner, 1994). This is possibly also true for cuttlefish. Cutaneous oxygen exchange is likely in all coleoids given that aerobic muscle fibers and dense capillary beds are located on the surfaces of the mantle muscle and not in its core (Bone et al., 1981). Many basic things about cephalopod blood and circulation are not properly quantified, including blood viscosity. Given the complex interaction of the Bohr effect, regional tissue hydrogen concentrations and cutaneous oxygen extraction, cephalopod blood and circulation may be better adapted for oxygen delivery than previously thought.

My speculation about cardiovascular function would not be complete without revisiting the role of venous contraction. Venous contraction may only be important for venous return in resting coleoids. During exercise, more important venous pressures may be created in a more "vertebrate" way, by vessel compression in the mantle. Besides helping venous return in resting coleoids, contractions of the anterior vena cava might also help control blood volume. The anterior vena cava is a distendable vein, well suited to blood storage (Shadwick and Nilsson, 1990). It may perform an analogous role to the liver in humans (Guyton, 1991), holding blood that is not needed in circulation. By

controlling the magnitude of anterior vena cava contractions and the aperture of the Wells valve (see chapter 2), the amount of blood that remains in the anterior vena cava and the amount that is driven into circulation, could be regulated.

5.3. The promise of non-invasive imaging technologies for the study of cephalopod circulation

Until now, we have been able to examine only cardiac function in cuttlefish. Non-invasive ultrasound has allowed us to examine cuttlefish vasculature for the first time. Coupled with synchronized videotape of cuttlefish behaviour, ultrasound has yielded new information about cuttlefish circulation, and furthermore how circulation, ventilation and locomotion may be interrelated. Ultrasound may also be applied to other coleoids that will remain still for periods of 30 s or more. However, the biggest hurdle to understanding cephalopod circulation is understanding blood flow in the periphery. MRI has been used to quantify blood flow in crustaceans (Bock et al., 2001), and could be used in cephalopods as well. The new use of non-invasive techniques such as ultrasound, Doppler and MRI in coleoid cephalopods may help to clarify the extent to which the cephalopod system can function so much like a fish or mammalian system and yet be so different.

5.4. Epilogue and contributions to scientific knowledge

The coleoid cephalopods are alluring to study because they are charismatic, vertebrate-like and have a sense of legend about them stemming from ancient stories of sailor-eating kraken. Contrary to what legends would have us believe, however, cephalopods are fragile animals to keep and culture in captivity. In temperate climates

such as Canada's, it is difficult to find the tiny live crustaceans that hatchling cephalopods require for food. Consequently, colonies are prone to failure. The adults have delicate skin and are prone to disease. There is a careful balance between ensuring there is always enough food for these highly active creatures but that the food does not linger in the tanks, breeding fatal bacteria, and that the tanks are scrubbed often enough to avoid outbreaks of disease but that the animals are not stressed from frequent tank disturbances. The food and labour that cephalopods require make them expensive. As a result of these and other factors, the supply of cuttlefish during my degree was tenuous and my access to squid limited. Consequently, the content of my thesis chapters may seem somewhat unrelated. However, each was undertaken with the overall objective of quantifying the changes in ventilation rate and heart rate that accompany preparation for fighting and fleeing in coleoids. While chapters 2 and 4 do not meet this objective directly, they provided the necessary comparison information about resting ventilatory and cardiac function in cuttlefish, and the methods to induce fighting in squid for future studies that may investigate its physiological correlates.

5.5.1. Original contributions to scientific knowledge

Physiology:

- Development of ultrasound to non-invasively monitor cardiovascular and ventilatory function in cuttlefish.
- Evidence that the anterior vena cava contracts peristaltically *in vivo.*
- Evidence that the lateral venae cavae contract peristaltically and that the efferent branchial vessels contract *in vivo.*

- Functional and histological evidence for a previously undescribed valve (the Wells valve) between the AVC and the branch point.
- Evidence that the mantle does not contribute to venous return through the pressures generated during ventilation.

Behavioural Physiology:

- Elucidation of the four stages of cuttlefish response to sudden visual stimuli.
- Evidence that cuttlefish heart rates drop after a sudden visual stimulus.
- Evidence that cuttlefish typically hyperinflate their mantles after sudden visual stimuli.
- Evidence that hyperinflation and decreased heart rate are interrelated, but not related to the chromatic and textural response (Deimatic Display).
- Hypotheses that a) decreased heart rate in cuttlefish after sudden stimuli is related to compression of the mantle capillaries. Mantle capillaries could be compressed by the radial muscle contraction that causes mantle hyperinflation; b) hyperinflation helps cuttlefish prepare for sudden escape jetting, should it be necessary; c) hyperinflation also decreases the need of cuttlefish to ventilate, increasing cuttlefish stillness and enhancing cuttlefish attention.

Behaviour:

- Confirmation that male *Loligo pealeii* use egg mops to regulate agonistic behaviour.
- Evidence that touching egg mops is necessary and sufficient to increase male agonistic behaviour.

- Evidence that sight is important in attracting squid to the egg mops and in maintaining agonistic behaviour between egg mop touches.
- Quantification of the duration of agonistic behaviour after egg mops have been removed from tanks containing paired male squid.

Extrapolation from chapters 2 and 3:

- Hypotheses that a) the regular contraction of the radial muscles aid peripheral circulation in resting cuttlefish; and b) the alternating contractions of the radial and circular muscles during jetting contribute importantly to circulation in coleoid cephalopods.

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Appendix 1: List of Videos on Accompanying CD

Video 2.1. Sonogram of a midsagittal section through the anterior vena cava, branch point, ventricle and mantle. For organ identification, see the legend in Fig. 2.3.B. NB Videos are rotated 180° from their still counterpart. The peristaltic contractions of the anterior vena cava (AVC) had the same rate as the contractions of the mantle (m), but were out of phase with them. The anterior vena cava had a different contraction rate than the branch point (BP) and the ventricle (V). The branch point and the ventricle, however, shared the same contraction rate.

Video 2.2. Sonogram showing a transverse section through the branch point, the efferent branchial vessels, the gills, and the mantle. For organ identification see the legend in Fig. 2.3.C. NB Videos are rotated 180° from their still counterpart. Notice how the gills move back and forth, but do not appear to contract. The branch point (BP) and the left efferent branchial vessel (EBV), however, contract noticeably at about the same rate, but out of phase with each other.

Video 3.1. An example of the behavioural video taken of a cuttlefish responding to a sudden visual stimulus. Notice the four locomotory stages of the reaction (described in Table 3.4.), the postural changes (widening of mantle and arms), and the chromatic and textural changes (components of the Deimatic Display). This cuttlefish did not show pronounced Mantle paling.

Video 3.2. An example of a sonogram of the collar flaps before and after presentation of a sudden visual stimulus. For identification of important postural parameters, see Fig. 3.2.B. NB Videos are rotated 180° from their still counterpart. The white object moving in space "d" is the collar flap. The medial, circular pulsating structure is the anterior vena cava. This trial is presented graphically in Fig. 3.5.A.

Video 3.3. An example of a sonogram of the ventricle before and after presentation of a sudden visual stimulus. For identification of important postural parameters, see Fig. 3.2.C. NB Videos are rotated 180° from their still counterpart. Notice how all contractions stop during hyperinflation (hyperinflation is the increase in area "f"), and then resume as hyperinflation subsides.

Video 5.1. Sonogram of a transverse section through the anterior vena cava and collar flaps of a mating female cuttlefish. Notice how the anterior vena cava maintains a steady contraction rate while the mantle contracts vigorously and more rapidly. After vigorous contractions cease, the collar flaps beat twice for every contraction of the anterior vena cava. The vigorous mantle contractions are caused by the arms of the male cuttlefish irritating the collar flaps of the female as they mate head to head.

All videos are in mpeg format.