# Innervation of ascidian siphons and their responses to stimulation

**G.O. Mackie, P. Burighel, F. Caicci, and L. Manni**

**Abstract:** The distribution of sensory cells and nerves was studied in the siphons of *Corella inflata* Huntsman, 1912 and *Corella willmeriana* Herdman, 1898 by immunohistology and electron microscopy. Each siphon has about 8000 primary sensory neurons. A coronal organ of the compound type is present on the oral tentacles. Convergence in the afferent pathway is estimated at >10:1. A new category of cells associated with the velar sphincter muscle is described at the tentacle bases. Responses to stimulation were recorded using flow meters. Both siphons are sensitive to touch and near-field vibrations. Removal of the oral tentacles did not diminish vibration sensitivity. Gentle stimulation of the oral siphon evokes crossed responses in which the atrial siphon closes and the velar sphincter contracts. Stronger stimulation produces squirts with closure of both siphons and branchial ciliary arrest. Experiments with polystyrene beads show that the oral tentacles are sensitive to contact with inflowing particles. Beads of 500–600 μm diameter evoked rejection responses 88% of the time, 355–425 μm beads 61%, and beads <125 μm less than 8%. These responses, attributed to the coronal organ, were lost after amputation of the tentacles. Electrophysiology confirmed that crossed responses and squirting are centrally mediated reflexes, but local conduction pathways also exist and survive deganglionation.

**Résumé :** Nous avons étudié par immunohistologie et microscopie électronique la répartition des cellules et des nerfs sensoriels dans les siphons de *Corella inflata* Huntsman, 1912 et de *Corella willmeriana* Herdman, 1898. Chaque siphon contient environ 8000 neurones sensoriels primaires. Il y a un organe coronal de type composé sur les tentacules oraux. Nous estimons la convergence dans la voie afférente à >10:1. Nous décrivons une nouvelle catégorie de cellules associées au muscle du sphincter du vélum à la base des tentacules. Nous avons enregistré les réactions à la stimulation à l'aide de débitmètres. Les deux siphons sont sensibles au toucher et aux vibrations dans l'espace adjacent. L'ablation des tentacules oraux ne diminue pas la sensibilité aux vibrations. Une stimulation douce du siphon oral provoque des réactions croisées de fermeture du siphon atrial et de contraction du sphincter du vélum. Une stimulation plus forte provoque des giclements avec une fermeture des deux siphons et l'immobilisation des cils branchiaux. Des expériences avec des billes de polystyrène montrent que les tentacules oraux sont sensibles au contact des particules qui entrent. Des billes de 500–600 μm de diamètre provoquent des réactions de rejet dans 88 % des cas, des billes de 355–425 μm dans 61 % des cas et des billes de <125 μm dans moins de 8 % des cas. Ces réactions, attribuées à l'organe coronal, sont perdues après l'ablation des tentacules. L'électrophysiologie confirme que les réactions croisées et les giclements sont des réflexes sous contrôle central; il existe aussi des voies de conduction locales et le phénomène continue après l'ablation des ganglions.

[Traduit par la Rédaction]

## Introduction

Evidence from classic studies on ascidian behavior makes it clear that the siphons are the regions most sensitive to tactile stimulation (Hecht 1918*a*, 1918*b*; Day 1919; ten Cate 1931), and sensory cells are known to be located around the rims of the siphons and in their inner and outer mantle epithelia (Fedele 1923; Millar 1953; Mackie and Wyeth 2000), although their distribution has never been exactly plotted. The sensory cells in question are primary sensory neurons, each with a single axon that goes to the brain. Until recently, and with a few rare exceptions, such cells were considered to be the only mechanoreceptive sensory cells to be found in adult ascidians.

A completely new sensory structure, the coronal organ, was recently described in the tentacles and velum of the oral siphon of the colonial styelid ascidians *Botryllus schlosseri* (Pallas, 1766) and *Botrylloides violaceus* Oka, 1927 (Burighel et al. 2003). Coronal organs have since been found in ascidians of all groups examined (Manni et al. 2004*a*, 2006). The organ is of exceptional interest because the sensory cells composing it are axonless, secondary sensory units that make both afferent and efferent synapses with neurons whose somata lie centrally. The cells are always ciliated, and in some cases the cilia are surrounded by clusters of stereovilli, as in the hair cells of the vertebrate acustico-lateralis system. Given the position of the Tunicata as an offshoot from the chordate line of evolution, the coronal organ may indeed represent an early expression of the same genes that gave rise to the acustico-lateralis system. The embryological evidence is consistent with such a derivation (Manni et al. 2004*b*; Mackie and Burighel 2005).

The coronal sensory cells of different ascidians vary con-

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siderably in the details of their apical structures. There may be one cilium or two, and the cilia may lie centrally or eccentrically within a cluster of stereovilli. The stereovilli may be of equal length, or graded in length from one side to the other, as in typical vertebrate hair cells. In *Styela plicata* (Lesueur, 1823) for example, two sorts of sensory cells occur in adjacent parallel rows. One sort has a single cilium and short stereovilli of equal length. In the other sort, there are two cilia situated within a crescent of stereovilli showing polarized length gradations, and the cells are oriented consistently with the longer stereovilli on the side facing the inward water stream. Such cells clearly qualify for the designation ''hair cell''. These and other variations suggest that coronal sensory cells have undergone functional specialization in a number of different directions within the Ascidiacea, as indeed have hair cells in vertebrates. However, there is virtually a complete lack of evidence regarding the function or functions of coronal organs at the present time.

In this paper we investigate siphonal sensory structures and sensitivity, including the role of the coronal organ in *Corella inflata* Huntsman, 1912. A major objective of the physiological work has been to determine if the oral tentacles (where the coronal organ is located) are sensitive to vibrations or tactile stimulation. We find that they probably function simply as mechanoreceptors which monitor particles coming in with the incurrent water stream.

## Materials and methods

Specimens of *C. inflata* were collected from beneath floats and docks in the waters around Victoria, British Columbia, and Friday Harbor, Washington, and maintained in sea-water aquaria at the University of Victoria and at the Friday Harbor Laboratories, University of Washington. The sea water at the Friday Harbor laboratories, where most of the feeding tests were carried out, is pumped from 12 m below the zero tide level. It passes to a header tank, from which it is fed by gravity to the laboratories. The water is not filtered, although heavier particulates settle out in the header tank. The water contains a fair amount of fine particulate matter that is sufficient to meet the needs of filter feeders such as *Corella*, which can be grown from the larval stage in the tanks without additional food. The system is thus optimal for experiments on feeding-related activities. Fecal matter tends to accumulate in the atrial cavity if the animals are left lying on their sides and it was found helpful to suspend them upside down from styrofoam floats, or to keep then in motion by circulating the water in their container.

Unless otherwise mentioned, all observations and illustrations refer to *C. inflata*. The distribution of nerves was also examined in *Corella willmeriana* Herdman, 1898 obtained from Bamfield Inlet, British Columbia. No significant differences from *C. inflata* were observed. A stolidobranch ascidian *Styela montereyensis* (Dall, 1872), from the open coast near Jordan River, British Columbia, was used for the experiments illustrated in Figs. 8B and 8C. Specimens were obtained through WestWind Sealab Supplies, 434 Russell Street, Victoria, BC V9A 3X3 (wwsealab@islandnet.com).

### Immunohistology

Animals were anesthetized in 0.02% MS 222, removed from their tunics, dissected, and pinned out flat with cactus spines in Sylgard®-lined petri dishes. Fixation was in 4% paraformaldehyde made up in 0.1 mol/L of phosphate-buffered saline (PBS). The tissue was generally fixed for 1–3 h, but longer periods gave equally satisfactory results. Preparations were washed in PBS containing 0.1% sodium azide and 0.25% Triton X-100. Primary antibodies used included anti-tubulins (12G10 and E7) from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, and anti-Tunicate I gonadotropin releasing hormone (tGnRH-I) from N.M. Sherwood, University of Victoria. Alexa 488 or 568 labelled secondary antibodies were used to visualize immunoreactive sites.

### Electron microscopy

Pieces of tissue from animals anaesthetized with MS222 were pinned out with cactus spines and processed for scanning and transmission electron microscopy (SEM and TEM, respectively). Some pieces were fixed in 2.5% glutaraldehyde in 0.4 mol/L of Millonig's phosphate buffer (1–2 h at room temperature), followed by post fixation in 1% osmium tetroxide in phosphate buffer. Others were fixed in 1.5% glutaraldehyde buffered with 0.2 mol/L of sodium cacodylate, at pH 7.4, plus 1.7% NaCl, and washed and post fixed in 1% osmium tetroxide in 0.2 mol/L of cacodylate buffer.

Following post fixation, pieces of tissue were dehydrated and embedded in Araldite®. Thick sections (1 μm) were counter-stained with toluidine blue. For TEM, thin sections (60 nm) were stained with uranyl acetate and lead citrate to improve contrast. Micrographs were taken with a Hitachi H-600 electron microscope operated at 75 kV.

Tissue pieces for SEM were fixed by the phosphate-buffered procedure described above, after which they were dissected, dehydrated and critical-point dried, sputter-coated with gold, and observed under a Cambridge Stereoscan 260.

### Behavioural physiology

Animals were studied in a perfusion chamber (capacity 360 mL) through which sea water was kept flowing at a constant rate (ca. 150 $mL \cdot min^{-1}$), sufficient to keep the water at a constant temperature and never above 15 °C. Under these conditions animals exhibited normal pumping behaviour and siphonal responses, and showed no prolonged contractions or lengthy cycles of spontaneous ciliary arrests of the sort seen in animals kept under less than optimal conditions. The animal was kept in position by one or two minute stainless steel pins inserted through the outer edges of the tunic, without piercing the mantle, into the Sylgard® lining the bottom of the perfusion chamber. The chamber was placed on the stage of a dissecting microscope, which allowed responses to be observed while recordings were being made. It was possible to position the animal in such a way that one could look directly in through the oral siphon and see the oral tentacles, and stimulate them and other parts selectively (Fig. 1A). A fibre optic light pipe was used to illuminate the animal from the desired angle.

Water-flow velocity through the siphons was recorded with thermistor flow meters (Mackie and Singla 2003). In most experiments, two flow meters were used, one for each siphon. Flow-meter recordings allowed both siphon contrac-

**Fig. 1.** (A) View into the siphons of a living *Corella inflata* showing the oral tentacles projecting into the lumen of the oral siphon (os). Touching them with a needle (*n*) evoked a partial contraction of the atrial siphon (as), referred to as the "crossed response". The oral siphon stays open. (B) Oral tentacles (*t*) and velar sphincter (sp) in a whole-mount preparation labelled with anti-tubulin antibody to show nerves. A substantial nerve bundle (arrowheads) runs along each side of the tentacle under the coronal organ. (C) Edge of tentacle shown in profile, with cilia of coronal organ and subcoronal nerve bundle (arrowhead) shown with tubulin labelling.

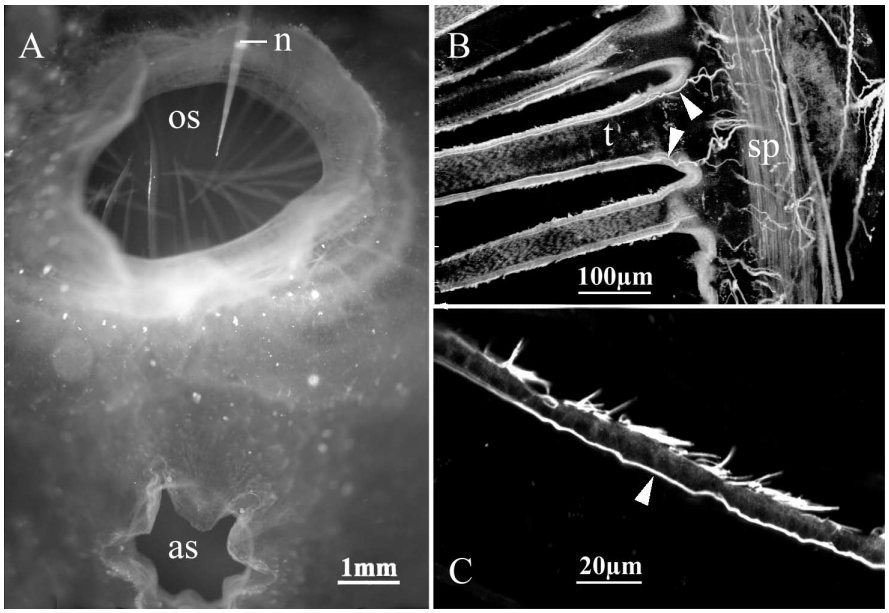
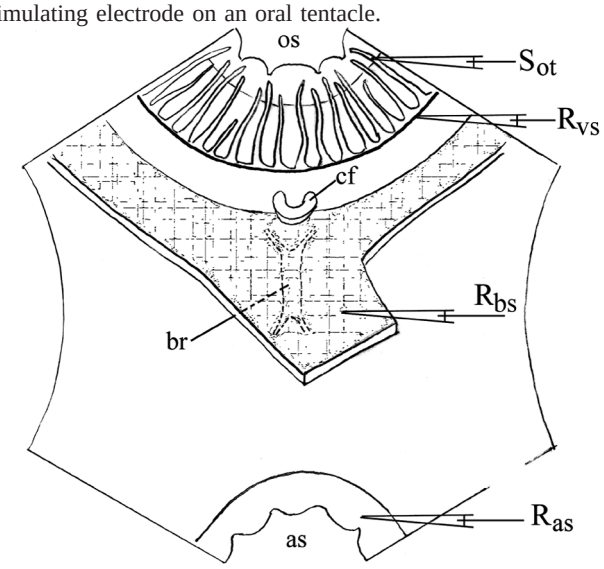



**Fig. 2.** Semi-intact preparation of *C. inflata* as used for electrophysiology. After removal of the tunic, the top of the animal was cut off and pinned out, with the internal organs facing up. Most of the branchial sac has been removed, but a (transparent) flap has been kept for attachment of an electrode. as, atrial siphon; br, brain (under branchial sac); cf, ciliated funnel; os, oral siphon; $R_{as}$, recording electrode on atrial siphon; $R_{bs}$, recording electrode on branchial sac; $R_{vs}$, recording electrode on velar sphincter; and $S_{ot}$, stimulating electrode on an oral tentacle.




tions and ciliary arrests to be monitored without any additional devices, but electrical recordings were also made from the siphons and the branchial sac using polythene suction electrodes attached to tissue surfaces. In the case of the branchial sac, a window was cut through the mantle to allow the electrode to be attached directly to the ciliated epithelium. Signals were amplified using capacity-coupled preamplifiers and displayed, along with data from the flow meters, on a laptop computer, using an AD Instruments PowerLab data acquisition system. Electrical stimuli were delivered either through fine metal electrodes or through polyethylene suction electrodes similar to those used for the recordings. Tactile stimuli were applied with a fine tungsten needle. *Corella* is so sensitive to vibration and touch that it is hard to stimulate different regions in a precise and consistent way with a hand-held probe. Accordingly, the stimulating needle was mounted on a 0.2 W loudspeaker, driven by current pulses from a Grass S48 stimulator and positioned using a micromanipulator. The amplitude of the deflections produced at the tip was controlled by adjusting the voltage setting on the stimulator. Pulse duration was kept at 0.5 ms.

For more detailed analysis of electrophysiological responses, a semi-intact preparation was used (Fig. 2). The animal was removed from its tunic. This included removing the thin layer of tunic that lines the siphons close to their outer edges. The top of the animal including the siphons and the brain was cut off and pinned out with the tunic side down, exposing the oral tentacles, nerves, and muscles from the inside. The siphons were incised on one side, allowing them to be pinned out flat. Enough of the branchial sac was retained to allow recording of ciliary arrests. Such preparations were made under MS 222 anaesthesia and were then returned to sea water for at least 5 h before use.

To determine whether the animal was sensitive to waterborne vibrations, a stainless-steel needle with a 1 mm diameter spherical glass ball on its tip was mounted on the loudspeaker in place of the tungsten needle used for tactile stimulation. The tip of this probe was placed close to or just inside the siphon opening. Pulses were delivered to the speaker coil at frequencies between 1 and 150 Hz for

a standard period of 300 ms. For each selected frequency, the minimum voltage producing a response was recorded as a measure of the threshold sensitivity value for that frequency. In practice, these values generally lay between 2 and 10 V. For these tests, no portion of the probe was allowed to make contact with any part of the animal or of the perfusion chamber, and precautions were taken to ensure that vibrations were not transmitted through the substrate.

Tests were carried out to see if the animal could detect particles entering the animal with the incurrent water. Test animals were placed in the perfusion chamber and their responses observed and recorded as described above. Particles were initially made by grinding up marine algae (*Chondracanthus* Delaroche, 1811, *Ulva* L., 1753) and separating out fragments in various size ranges by filtration and centrifugation. Living *Isochrysis* and *Isochrysis* suspensions made from frozen concentrates (from Brine Shrimp Direct, Ogden, Utah) were also used. For more critical tests, polystyrene and Sephadex® beads of known size ranges were chiefly used. As Sephadex® beads swell in water, they were allowed to swell completely before being used and the diameters given are for the swollen beads. Tests were carried out by loading a suspension of the particles into a long-nosed Pasteur pipette with a length of flexible rubber tubing attached to its stem. The tubing was connected to a syringe by a three-way stopcock. The pipette was positioned at a slightly inclined angle so that its opening lay close to, or just above, the oral siphon, within the incurrent water stream. Slight pressure was applied to the tube, propelling particles along the pipette and out its tip. It was possible to deliver larger particles one or two at a time, monitoring the process visually to ensure that the particle entered the oral siphon, and noting any response. For each size of particle in the tests summarized in Fig. 10, passage of approximately 100 beads into the siphon was observed.

For removal of the oral tentacles, animals were opened on their left sides to create a small window through the tunic, mantle, and branchial sac close to the oral siphon, and the tentacles were then removed from inside with iridectomy scissors. It was possible to remove 80%–90% of each tentacle, but not the whole structure. The tentacle stumps and velum remained.

In experiments involving brain surgery, animals were anaesthetized with MS 222 and the brain was exposed by dissection from the outside, midway between the two siphons. It was not possible to remove the brain without also removing the neural gland, but apart from some blood loss, the operation did little other damage. Animals regained some responses after 5 h, but were generally left until the next day. At the end, the operated regions were examined histologically to make sure all the brain tissue had been removed.

## Results

### Sensory structures and their innervation

#### *Siphons*

The distribution of primary sensory neurons in the epithelia covering and lining the siphons was chiefly studied using tGnRH-I antiserum, which binds to the cell membranes of these neurons without the extensive background labeling of epithelial cells associated with anti-tubulin (Figs. 3A, 3B, 3E, 3F, 3G). The significance of GnRH-like immunoreactivity in primary sensory neurons in *C. inflata* is unclear. It has been suggested that it acts as a neuromodulator; however, no such immunoreactivity was observed in another species, *Corella eumyota* Traustedt, 1882 (Mackie and Singla 2004). The sensory cilia, shown well with anti-tubulin (Figs. 3C, 3D), are not shown with the tGnRH-I label. The cells characteristically occur side by side in pairs (doublets), with their membranes pressed close together, and the zone of apposition appearing as a bright line in figures such as Fig. 3E. They form a continuous row around the rims of both siphons (Figs. 3B, 3C), except in the regions adjacent to the ocelli. In the outer mantle epithelium under the tunic, the doublets are abundant close to the rims, but their numbers taper off down the sides. There are about 3500 cells on the outside.

On the inside of the oral siphon, there are about 4750 cells located within a sensory field that occupies the outer third of the space between the velum and the rim. (The distribution in the atrial siphon is similar, although there is no velum.) The great majority of the bright spots seen in Fig. 3A would be doublets, like those shown enlarged in Fig. 3E. The lower border of the sensory field is a clearly defined line demarcating sensory and non-sensory zones (white arrowheads in Fig. 3A). Primary sensory somata are completely absent in the latter, although the axons from the cells in the sensory zone run back through this region. Along this border line, rounded axonless cells are frequently seen (Fig. 3H), along with what appear to be early stages in the formation of sensory neurons, with very short axons (Figs. 3F, 3G, 3H). We suggest that the rounded axonless cells are neuroblasts and that sensory neurons form in this region as the animal grows, but this requires experimental validation. These presumed stages in neurogenesis are very strongly GnRH-immunoreactive (ir) positive, more so than the mature cells with long axons. Similar, rounded, strongly GnRH-ir cells have been described in the dorsal blood sinus associated with the dorsal strand plexus and cupular strand sense organ (Mackie 1995; Mackie and Singla 2004) and are likewise implicated in neurogenesis.

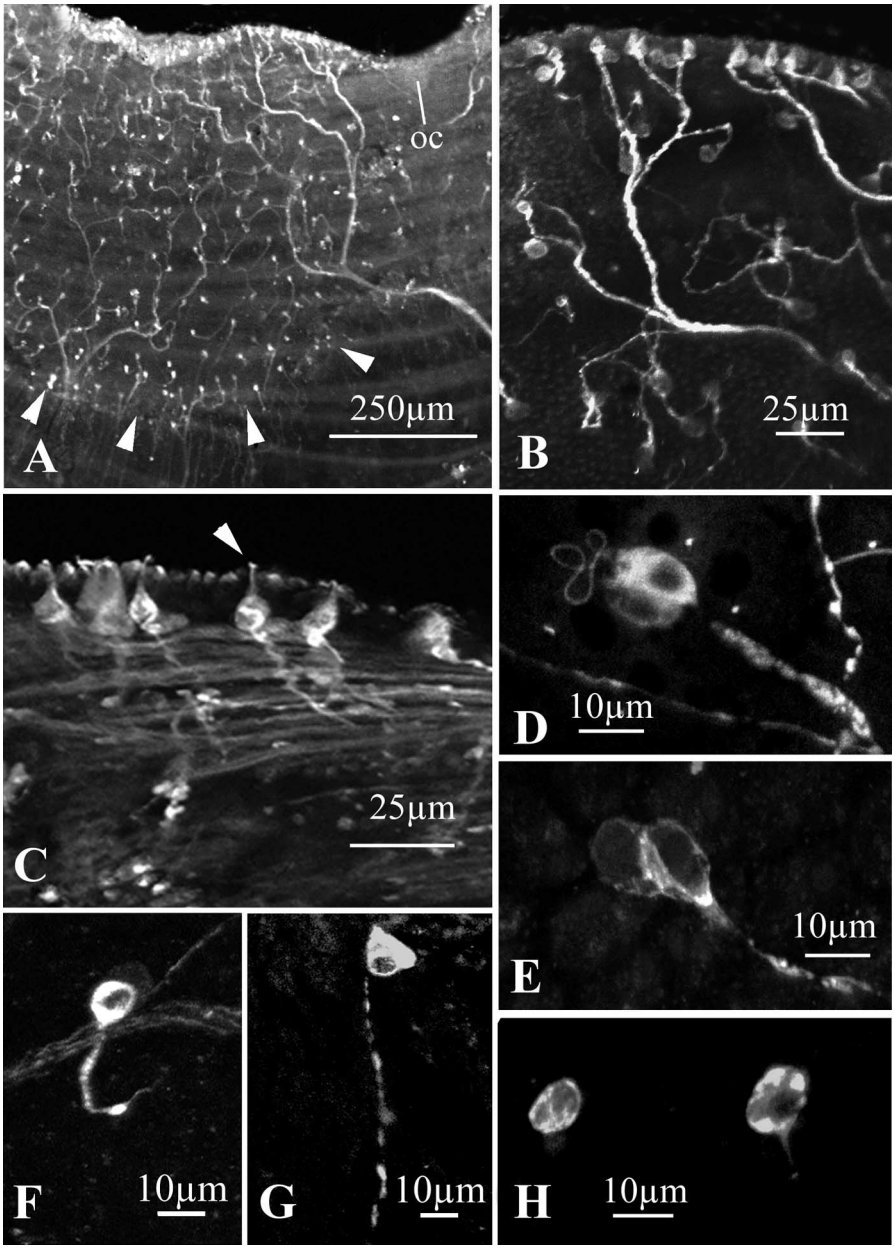
It is also noteworthy that the general epidermal cells lining the siphons differ in the two zones. Those in the sensory zone have short cilia, possibly equivalent to the ''primary cilia'' that characterize many vertebrate epithelia (Salisbury 2004), while those in the non-sensory zone lack them. In the former, the microtubular array in the cytoplasm is focussed on the ciliary bases. There is no such polarity in the latter.

The general distribution of primary sensory cells is summarized diagrammatically in Fig. 4A. It is noteworthy that the areas where the sensory cells are located, both inside and outside the siphon, are covered by tunic, although the inner tunic layer is very thin. The sensory cilia may actually penetrate the tunic and, in some regions, break off when the tunic is removed, as there are many rather short cilia (<5 μm) particularly near the siphon rims. Further back, some cilia are much longer (<15 μm).

#### *Oral tentacles*

The tentacles, velum, and coronal organ in *Corella* resemble those described in *Chelyosoma productum* Stimpson, 1864 (Manni et al. 2006). When the oral siphon is open,

**Fig. 3.** Primary sensory neurons in the siphons of *C. inflata*. The tunic has been removed in all cases. (A) Inner mantle epithelium (tGnRH-I labelling) showing scattered sensory neurons (bright spots) and their axons running back and entering branches of nerves stemming from the brain. Sensory neurons are most abundant around the upper rim of the siphon (top of picture) except close to the ocellus (oc). The lower limits of the sensory field are marked by arrowheads. (B) Detail of siphon rim (tGnRH-I). (C) Sensory neurons at the rim in a tubulin-labelled preparation, showing short sensory cilia (arrowhead). (D) Tubulin-labelled sensory neuron doublet showing long cilia. (E) Similar doublet with tGnRH-I label. The label appears to be restricted to the cell membranes of the cell body and axons, and the cilia do not show. (F), (G), and (H) show tGnRH-I labelled cells at the line of demarcation between sensory and non-sensory zones (arrowheads in Fig. 3A). The cells label more intensely than mature sensory neurons. Some lack axons, while others have what are interpreted as growing axons.

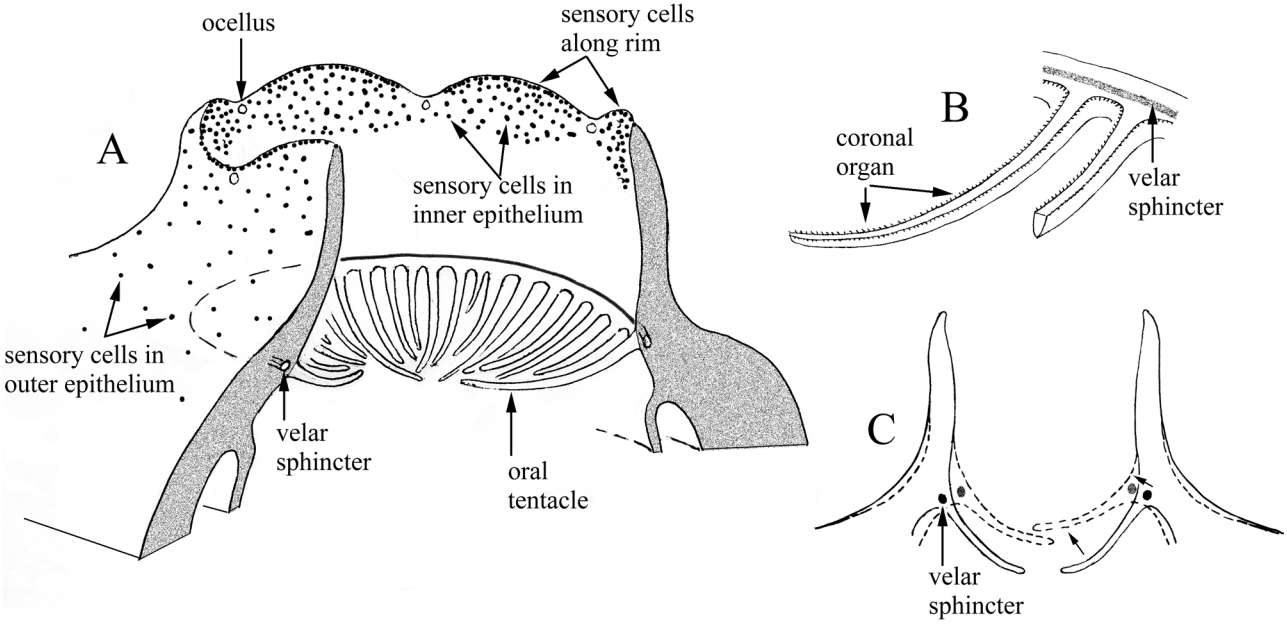



the tentacles are readily seen projecting into the lumen (Fig. 1A). There are about 30 tentacles in a full-grown animal, ranging in length from 0.5 to 2.0 mm, with the majority falling into the 1.0–2.0 mm range. In life, they are roughly triangular in cross-section (Fig. 4B), but shrinkage and distortion during fixation may cause a median fold to appear. In the relaxed condition, the tips of the longer tentacles meet in the centre of the lumen (Fig. 4A), but when the velar sphincter contracts, the tentacle tips come closer together thus reducing the gaps through which particles must pass (Fig. 4C). The tentacles taper toward their tips (Fig. 4B). In the relaxed state, the gaps between the tentacles are about 450 µm near their bases, while at their tips few gaps wider than 200 µm are seen. When the sphincter contracts (Fig. 4C), all these gaps are probably reduced by at least 25%, but this was hard to measure accurately.

### *Innervation of the tentacles and velar sphincter*

A rich nerve plexus is present in the area of the velar sphincter muscle (Fig. 1B) and adjacent body-wall muscles,

**Fig. 4.** (A) Oral siphon of *C. inflata* cut open to show internal structures. The tunic is not shown but covers dotted areas. The dots represent sensory cells and indicate relative density and distribution, not size or absolute numbers. (B) Detail of tentacles (one cut short) showing cilia of the coronal organ. (C) Role of velar sphincter. When it contracts, it elevates the tentacles and brings them closer together.




with substantial nerve tracts coming together and running back toward the brain. It is not possible to distinguish sensory and motor components within the plexus, but conspicuous bundles of neurites run from it into the tentacle lumen, one on each side beneath the coronal organ. Some of the units composing these bundles are presumably the dendrites of sensory afferents whose cell bodies lie in the brain. The coronal organ is visible by virtue of its sensory cilia, which show well with anti-tubulin labelling. (Fig. 1C). The cilia are 10–12 μm long. The subcoronal nerve bundle can also be seen in this picture. In a tentacle 1 mm long, we estimate that there are ca. 400 coronal cells, but only a tenth of that number of axons, some of which are presumably efferents. Thus, there must be a high degree of convergence, with 10 or more coronal sensory cells providing input to each afferent neuron.

There appears to be a second, independent innervation of the sphincter muscle from a cluster of GnRH-ir cells located at the tentacle base (Fig. 5). We will refer to these as "*b*" (basal) cells. The axons from the *b* cells run out along the sphincter muscle (Figs. 5A, 5B), and do not appear to associate with the nerves going to the coronal organ or with the general peripheral plexus located in the sphincter region. In a fully grown tentacle, there may be as many as thirty *b* cells, arranged in doublets, with their cell bodies closely apposed. Doublets are shown enlarged in Fig. 5C and in the inset in Fig. 5A. The nuclei are seen as dark areas where tubulin is absent. There is only one axon per cell. In one small (?immature) tentacle (Fig. 5B), only five doublets were counted, and in one of these, the axon from one member of the doublet ran up into the tentacle instead of down along the sphincter muscle. No other such examples were seen and we regard this as a developmental anomaly.

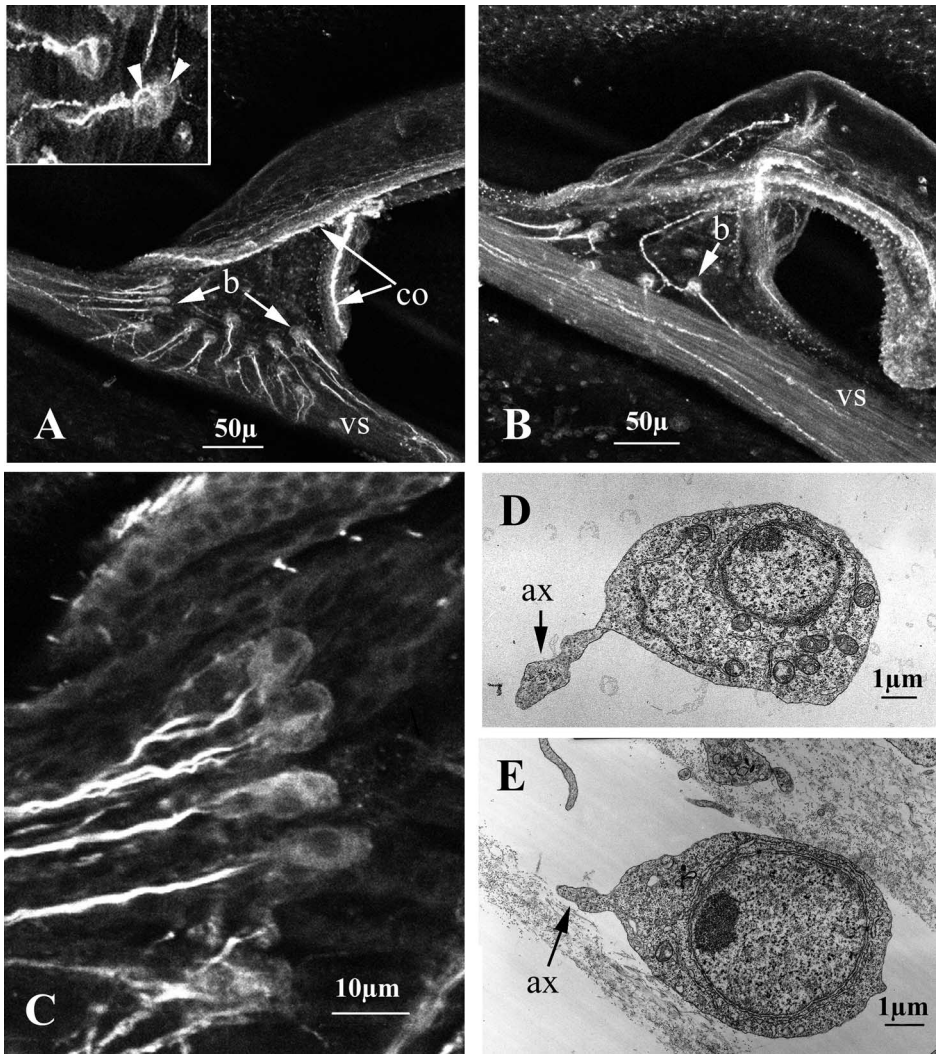
Seen with transmission electron microscopy, the *b*-cell bodies lie within the blood sinus at the base of the tentacle, sometimes close to the collagenous lining of the blood space, but often completely free, suspended from their narrow axonal necks. The cells are distinguishable from blood cells by their compact, rounded form and lack of filopodia and from the presence of an axon. They show no signs of having a secretory or phagocytic function and are quite clearly nerve cells, indeed they resemble primary sensory neurons in having an axon and occurring in pairs but, unlike the sensory neurons seen in the mantle epithelia, they lack cilia or any other obvious sensory component (Figs. 5D, 5E). SEM images of the surface of the tentacles in the areas overlying *b* cells showed no external sensory structures that could be associated with them.

### *Structure of the coronal organ*

The coronal organ consists of a row of sensory cells running along each side of a tentacle on its upper side, whose cilia project to the exterior and are clearly visible with tubulin labelling (Figs. 1, 5A) and SEM (Figs. 6A, 6B). In a thick section cut transversely through the tentacle (Fig. 6C), the two areas indicated by arrowheads are the coronal organs consisting of ciliated sensory cells and adjacent secretory cells, shown enlarged in a TEM (Fig. 6D). C-shaped supporting cells lie on either side. All the cells in the coronal organ are attached to one another by extensive apico-lateral tight junctions, but gap junctions have not been identified. Having both ciliated and secretory cells, the *Corella* coronal organ falls under the "compound" category, as described in the closely related *Chelyosoma* (Manni et al. 2006).

Two to four ciliated sensory cells are typically seen in cross sections. The cells are flask-shaped in profile (Fig. 6D) and have several cilia projecting from their tips, associated with prominent basal bodies and striated rootlets (Fig. 6E). Microvilli and stereovilli are absent. The apical surface lacks a glycocalyx, unlike the adjacent secretory and supporting cells. On the inner side, the coronal organ cells lie on a basal lamina, which is all that separates the

**Fig. 5.** The *b* cells of *C. inflata* shown with tubulin labelling (A–C) and transmission electron microscopy (D, E). (A) Tentacle base showing *b*-cell doublets (*b*). Their axons run out along the velar sphincter muscle (vs). The coronal organ cilia (co) are also visible. The arrowheads in the inset show the two cells composing a typical *b*-cell doublet. Each cell has one axon. (B) An atypical example of a *b*-cell doublet where the axon of one cell runs into the tentacle, the other to the velar sphincter (vs). (C) *b*-cell clusters enlarged. (D, E) Sections of *b* cells, showing a single axon and no obvious sensory specializations.




cells from the underlying blood sinus. All the sensory cells are infolded at their bases, forming a groove that extends along the entire coronal organ and contains a group of neurites. The latter are not clad in extracellular matrix material and the basal lamina does not extend into the groove, which allows the membranes of the neurites to be closely apposed to the basal membranes of the sensory cells in places. These sites frequently show synaptic specializations: paired thickenings of the apposing membrane and vesicles attached to the membrane on one side (Fig. 6F). Afferent synapses (with the vesicles on the sensory cell side) were fairly frequently observed, and a few efferent synapses were also seen. Axons were never seen emerging from the bases of the sensory cells, so we are dealing with secondary sensory cells as described in other ascidians (Burighel et al. 2003; Manni et al. 2004*a*, 2006). The neurites running in the basal groove and elsewhere in the tentacle blood sinus are less than 0.3 µm in diameter. They contain many microtubules, as well as some mitochondria and vesicles. Given the evidence of afferent and efferent synapses, it follows that some of the neurites are motor and others are sensory, but it was not possible to distinguish the two on the basis of their ultrastructure. With the exception of the *b* cells, no nerve cell bodies were observed in the tentacles. The sensory neurites associated with coronal organ presumably have their cell bodies in the brain, like the motor neurons.

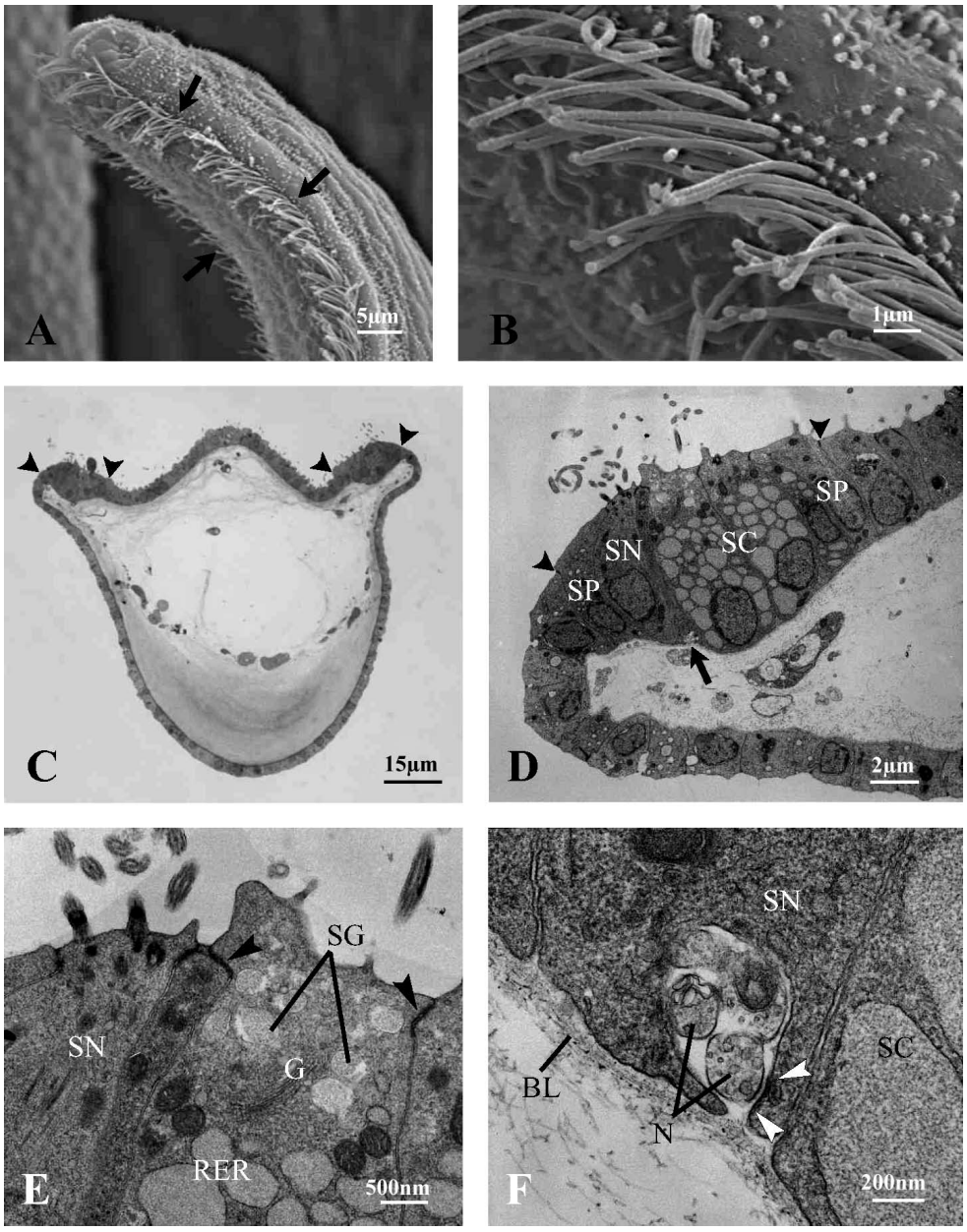
The secretory cells flanking the sensory cells contain a well-developed Golgi component, rough endoplasmic reticulum, and numerous, prominent secretory granules. Some of these appear to be associated with the apical cell membrane, preparatory to release. The secretory cells lack basal infoldings and do not appear to be innervated.

## Behavioural physiology

### *Normal activity in the aquarium*

Maintained in slowly flowing sea water at 12 °C, the animals showed no regular patterns of siphon contraction, squirt-

**Fig. 6.** Coronal organ structure of *C. inflata*. (A) Low-magnification SEM of a tentacle tip showing sensory cilia (arrows). (B) Sensory cilia enlarged. (C) A 1 μm section of a tentacle cut transversely. Arrowheads delimit the coronal organ on each side. The interior of the tentacle is a blood space in life, but the blood has coagulated and settled on the bottom, leaving an empty space in the middle. Blood cells are visible around the edges of this space. (D) TEM of coronal organ (delimited by arrowheads) showing secretory cells (SC), sensory neurons (SN), and supporting cells (SP). The arrow points to the basal infolding, which contains neurites. A vacuolated blood cell is seen within the blood sinus. (E) An enlargement showing apical ends of sensory and secretory cells. The latter have a well-developed supranuclear Golgi component (G), a rich rough endoplasmic reticulum (RER), and numerous secretory granules (SG). Several cilia are seen at the tip of the sensory cell (SN). (F) Basal portion of a sensory cell (SN) and adjacent secretory cell (SC) showing the basal infolding of the cell membrane of the sensory cell. Neurites (N) lie within the groove. The basal lamina (BL) does not extend into the groove. An afferent synaptic site is marked with white arrowheads.




ing, or ciliary arrest. Such events occurred infrequently and at irregular intervals, and were probably due to intake of large particles or to vibrations in the building. Flow-meter recordings from the oral siphon of an animal kept near an open window for several days in May 2003 showed an average of 5 squirts per hour and no obvious variations related to the alternation of light and dark periods over the 24 h cycle, but such observations have not been made on a regular seasonal basis.

### *Responses to stimulation of the oral siphon*

#### *Crossed response*

Light tactile, vibrational, or electrical stimulation of the oral siphon or the oral tentacles evokes the crossed response described by Hecht (1918*b*). This consists of one or a short series of contractions of the atrial siphon, while the oral siphon generally stays open or contracts only slightly.

**Fig. 7.** Differentiation of crossed and squirt responses in an intact *C. inflata*. The top two lines show changes in flow velocity through the oral and atrial siphons (os and as, respectively), while the third line shows ciliary arrests (ca) recorded electrically from the branchial sac. Responses were evoked by electrical stimulation, shock artefacts being shown on the bottom trace (st). (A) A weak shock evoked a crossed response (cr), while a stronger one evoked a squirt (sq) that was accompanied by ciliary arrest. (B) A crossed response on an expanded time scale. (C) Stimulation evoked a crossed response that was immediately followed by a squirt.

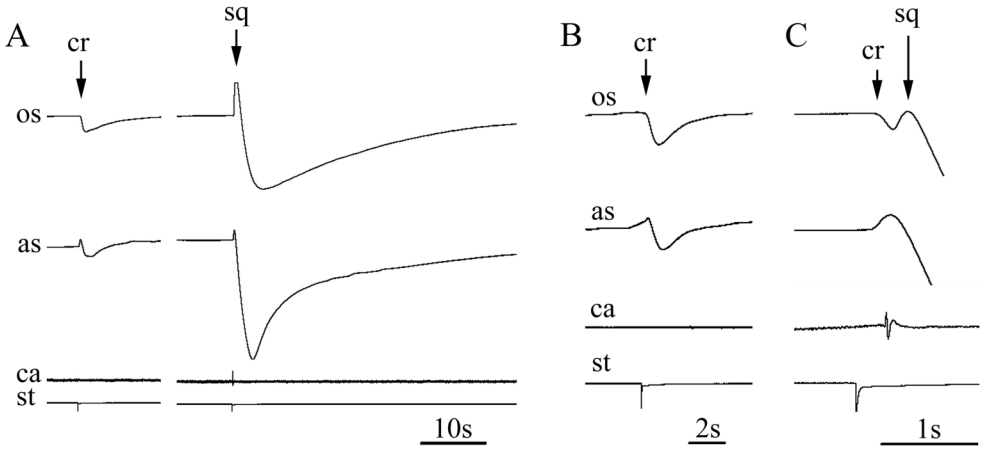



Figure 1A shows the appearance of the two siphons during a crossed response evoked by prodding the oral tentacles with a glass needle: the atrial siphon contracted to less than half its resting diameter and there was no change in the diameter of the oral siphon. The branchial cilia continue to beat steadily during the crossed response and, as the atrial siphon reopens fully after a few seconds, water flow through the animal continues with no more than a brief hesitation (cr in Fig. 7). Flow recordings during the crossed response show a brief upward deflection at the atrial siphon, followed by a downward deflection. The initial upward deflection reflects the sudden narrowing of the siphon aperture as the siphon contracts, causing a transient increase in the rate of flow out through it. The siphon then relaxes and the flow record shows a downward deflection.

Flow-meter recordings from the oral siphon during the crossed response show a simple downward deflection that represents a reduction in flow owing to contraction of the atrial siphon. The siphon itself does not contract, or contracts only very slightly, so there is no counterpart to the upward deflection seen at the start of the atrial response.

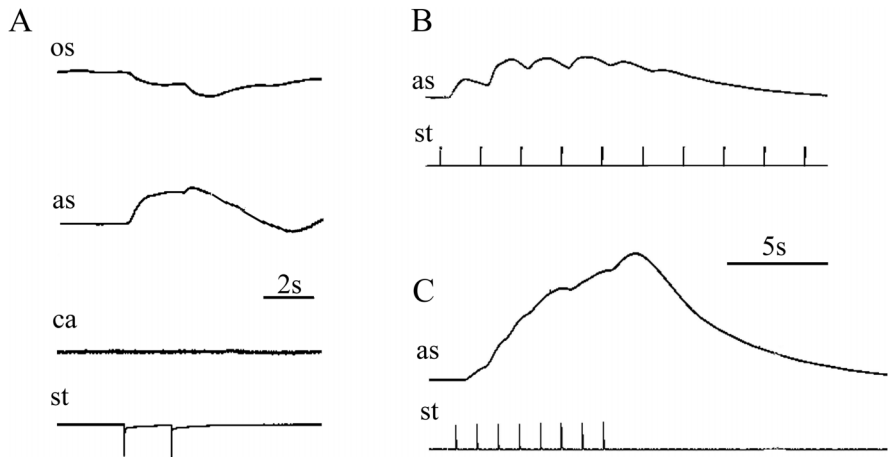
The degree of closure of the atrial siphon during the crossed response is variable, depending on stimulus strength and time since the last response. The response grades from a slight twitch of the siphon rim to almost complete closure. Larger responses are typically accompanied by a second effector response: the ring of tentacles in the oral siphon tightens, bringing the tentacle tips closer together, and reducing the gaps between them (shown diagrammatically in Fig. 4C). This occurs whether the stimulus is applied directly to the tentacles or to the inside or outside of the oral siphon. The response can also be evoked by a vibratory stimulus generated by a probe inserted into the siphonal opening, without touching the animal. These stimuli can all act to produce the crossed response in conjunction with the tightening of the tentacular ring and with little if any perceptible contraction of other regions of the oral siphon. The constriction and elevation of the tentacular ring is due to contraction of the velar sphincter, a prominent circle of muscle that runs around the siphon at the level of the tentacles. When it contracts it narrows the lumen in that part of the oral siphon, bringing the tentacle tips closer together while elevating them slightly (Fig. 4C). Sphincter contractions are graded according to stimulus strength. Stronger responses may be accompanied by slight contractions of the rim of the oral siphon, showing that excitation spreads throughout the siphon. Presumably the muscles in most regions have a higher response threshold than the sphincter and the sphincter response is simply the most sensitive indicator of general spread of excitation rather than being a categorically distinct motor component.

Sphincter contraction and consequent tightening of the ring of oral tentacles have occasionally been observed in isolation when large particles carried in with the incurrent water stream strike the tentacles; however, such events usually evoke the crossed response. As we note below, cutting the brain in half transversely makes it possible to study sphincter contractions in isolation.

All the responses described above are graded in amplitude according to the strength of the stimulus and inter-stimulus interval. The system that coordinates them also exhibits pacemaker capability, as a single shock can evoke not just one but a series of contractions. In such cases, the velar sphincter and the atrial siphon may continue to contract in unison. Similarly, repetitive stimuli evoke progressive increments of contraction in the atrial siphon and velar sphincter, producing ''staircase'' flow records. Staircase records were hard to obtain from *Corella* (Figs. 8A, 8B), as repetitive stimulation usually led to full-scale squirts, but summation was readily demonstrable in *S. montereyensis*, where it was possible to show that the slope of the staircase depends on the frequency of stimulation (Figs. 8B, 8C).

The oral tentacles, the inner surfaces of the siphon close to the rim, and the rim itself are the most sensitive regions, but the outer side of the siphon is also sensitive to touch, despite the thickness of the tunic. It was noted above that primary sensory neurons are distributed throughout the epithelium in these regions, but are absent in the inner part of

**Fig. 8.** Summation of crossed responses. (A) In *C. inflata*, shocks 2 s apart each evoked increments of contraction in the oral (os) and atrial (as) siphons. Lack of accompanying ciliary arrest (ca) shows that these responses were not squirting behaviour. (B, C) Summation of crossed responses recorded from the atrial siphon in *S. montereyensis*. The slope of the "staircase" depends on the frequency, i.e., 0.5 Hz in (B) and 1.0 Hz in (C). st, shock artefacts as described in Fig. 7.




the siphon where the tunic was absent. This zone is relatively insensitive to touch. However, the animal is so sensitive that touching almost any part of its body can evoke responses, presumably because vibrations are transmitted passively through the whole body, including the tunic and the blood vascular system.

*Squirt response*

While delicate stimulation of the inner surfaces of the siphon or oral tentacles evokes degrees of the crossed response, stronger stimulation evokes a strong, synchronous contraction of both siphons and adjacent regions of the body wall, accompanied by arrest of the cilia of the branchial stigmata that create the water current. Crossed responses do not grade into squirts, although they sometimes immediately precede and appear to trigger squirts. This is probably due to the mechanical disturbance caused by the crossed response exciting the animal's vibration sensors (Fig. 7C). Ciliary arrests were recorded with a suction electrode inserted through the mantle wall and attached to the branchial sac (Figs. 7A, 7C).

Flow records of squirts show a brief upward deflection, representing rapid emission of water from both siphons, followed by a marked, long-lasting downwards deflection, representing gradual diminution of flow following the ciliary arrest. The initial upward deflection is smaller at the atrial siphon than at the oral siphon, because water is already flowing in the outward direction there, whereas the flow direction is reversed at the oral. Repetitive stimulation causes a series of ciliary arrests and prolonged cessation of flow. Following a single stimulus, animals sometimes show series of synchronized ciliary arrests and siphonal twitches, exhibited in a regular rhythmic pattern, indicative of pacemakers in the control pathway (Takahashi et al. 1973; Mackie et al. 1974). Flow picks up again as the cilia start beating again, and this can occur before the siphons have reopened to their full extent.

***Responses to stimulation of the atrial siphon***

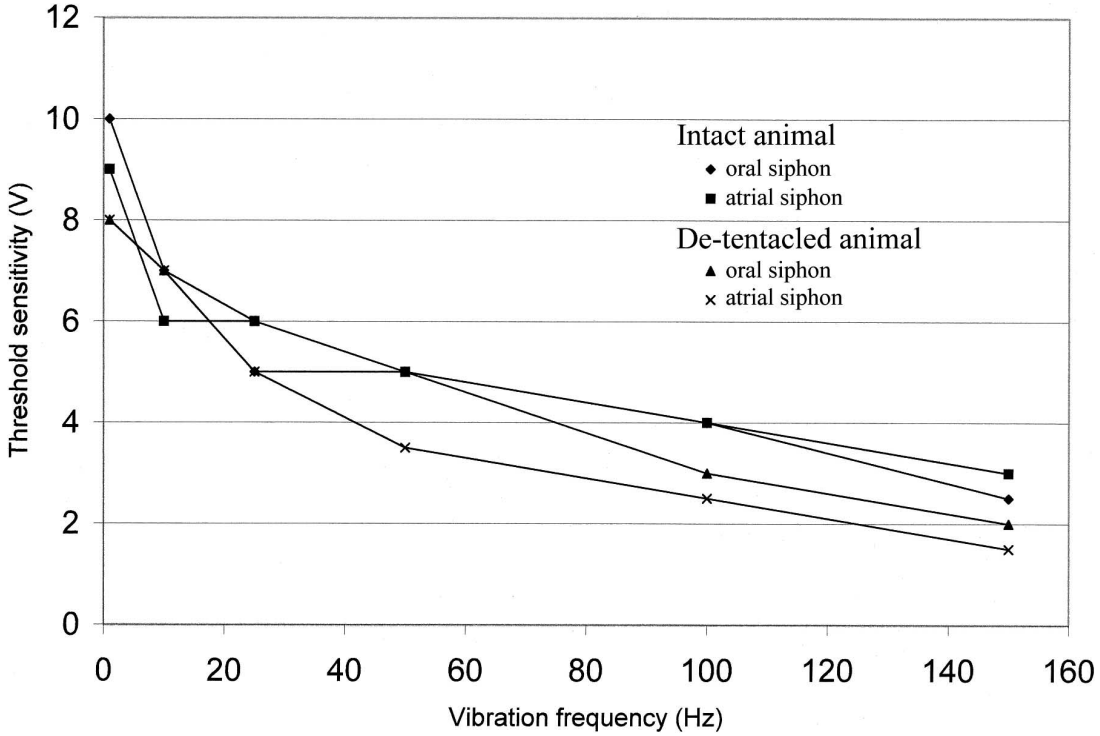
In intact animals, tactile or vibrational stimulation at the atrial siphon evokes one or a short series of contractions of the siphon itself, typically accompanied by contractions of the velar sphincter in the oral siphon. Thus, the crossed response evoked from this site appears similar to the response evoked by stimulation of the oral siphon. Stronger stimulation of the atrial siphon evokes the squirt response as described above for stimulation of the oral siphon, including the ciliary arrest component.

***Effects of brain surgery***

If the brain is completely removed, the normal crossed and squirt responses are abolished, although local contractions can still be obtained in the siphons and velar sphincter by local stimulation. These closures are slow and weak compared with the responses of intact animals, and reopening occurs immediately. The branchial cilia beat steadily and rarely arrest when the siphons are stimulated. In the case of the oral siphon, stimulation of the rim can still evoke sphincter contraction with little or no contraction of muscles in the intervening regions, although excitation must have passed through these regions, an observation that supports our earlier suggestion that muscle response thresholds vary markedly between the sphincter and other regions. Debrained animals continue to produce a mucus sheet from the endostyle, transport and ingest it, and pass food through the gut in the normal way. Defecation may be hampered by lack of squirting, which normally helps dislodge fecal pellets. The heart continues to beat and reverse direction periodically in debrained animals.

Where the brain is not extirpated but simply bisected transversely, the animal continues to show acute vibrational and tactile sensitivity; however, the responses of the two siphons are no longer coordinated or, if they appear to be, this is almost certainly attributable to vibrations transmitted passively through the body from a responding siphon to the other. The siphonal responses can be as strong and rapid as in intact animals, in contrast to the sluggish responses of debrained animals. We conclude that they are reflex responses involving pathways through the intact halves of the brain supplying the two siphons.

**Fig. 9.** Sensitivity to water-borne, near-field vibrations as described in the text.




***Responses to water-borne vibration***

We have noted that the animal responds to water-borne vibrations in exactly the same way that it does to direct touch. Vibratory stimuli were used in a number of experiments because they can be controlled precisely in terms of amplitude, frequency, and duration, and applied consistently without direct contact with the animal. Pulse trains of a few hundred milliseconds are more effective than single pulses. For instance, an animal that gave a threshold crossed response with a single pulse when the probe was 2 mm away responded to a 300 ms pulse train at 100 Hz when the probe was 4 mm away, the amplitude and duration of individual pulses being kept constant.

A series of tests were carried out to determine the threshold response levels at different oscillation frequencies of (*i*) the oral siphon, (*ii*) the atrial siphon, (*iii*) the oral siphon after truncation of the oral tentacles, and (*iv*) the atrial siphon after truncation of the oral tentacles (Fig. 9). It was not possible to remove the tentacles completely, but they were shortened down to stumps under MS 222 anaesthesia, after which the animals were allowed to recover overnight before testing. The results suggest firstly that over the range 1–200 Hz the two siphons respond similarly to vibratory stimuli and secondly that removal of the oral tentacles does little if anything to diminish responsivity.

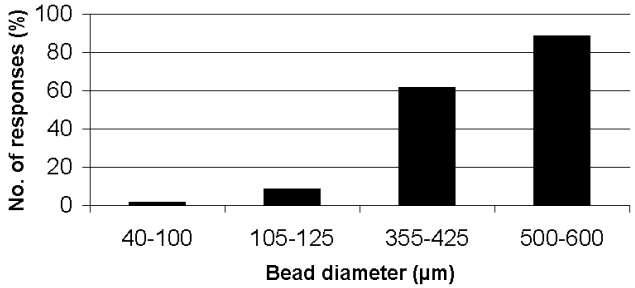
These results do not preclude a role for the oral tentacles as tactile or vibration sensors; indeed they are clearly sensitive to direct touch, but the results point to the primary sensory neurons located in the siphon walls as being the tactile and vibration sensors.

***Responses to particulates***

Tests with Sephadex® and polystyrene beads introduced into the incurrent pathway showed that animals with intact oral tentacles responded with crossed responses or squirts to the impact of larger beads on the tentacles. There was little response to beads below 125 μm diameter, but there was strong responsivity to individual beads in the 355–600 μm range (Fig. 10). Single beads (355–425 μm) usually evoked a crossed response, while several beads arriving together evoked squirting and expulsion of the beads. Beads which rolled in slowly along the inner wall of the siphon and landed on the tentacles gently often produced no response, unlike those carried in with the full velocity of the middle of the incurrent stream. The larger beads sometimes lodged in the tentacle mesh for several minutes without evoking responses, but eventually either slipped through the mesh or were ejected when the animal was caused to squirt by further bead impacts. Squirting was highly effective in dislodging material suspended in the tentacle mesh, while the crossed response was rather less effective. However, the movements of the tentacles during the crossed response and the accompanying slight hesitation in water flow sometimes served to shake previously trapped particles loose. In these tests, the velar sphincter was allowed to relax completely following each response since the drawing together of the tentacles reduced the apertures in the mesh, increasing the probability of bead impact. Nevertheless, it was frequently observed that crossed responses led directly into squirts (Fig. 7C).

After truncation of the oral tentacles, animals almost completely lost the ability to intercept and reject particles, including large bits of algal debris and detritus fragments several millimetres across. This material, which would have been rejected in an animal with tentacles, was drawn into the branchial sac, captured in the mucus sheet, and ingested along with the small particles that the animal normally collects.

**Fig. 10.** Responses of the oral tentacles to polystyrene beads of different sizes as described in the text.




Tests on intact animals with a suspension made from ground up and filtered *Ulva* fronds showed that the shape of the particle can be a factor, as thin flat fragments up to 800 µm wide readily slipped through sideways between the tentacles without causing responses. By contrast, relatively much smaller but chunkier fragments of *Chondracanthus* were detected and rejected. *Isochrysis* (ca. 5 µm diameter) were never intercepted by the tentacles and huge numbers of them could be rapidly collected, turning the branchial sac green. Squirting and ciliary arrests finally ensued when the *Isochrysis* laden mucus reached the languettes and started to be ingested. This probably corresponds to the "satiation response" of Van Weel (1940). It is not known how the animal senses satiation, but it is very unlikely that the oral tentacles are involved.

*Styela montereyensis* was not used extensively for tests on particulates, but in a few tests it appeared to behave similarly to *Corella*. Polystyrene beads in the 355–425 µm range were accepted if delivered one at a time, but clusters of them and larger beads (500–600 µm) evoked crossed responses or squirts. There were no responses to 105–125 µm polystyrene beads or to 40–120 µm Sephadex® beads.

Animals whose brains had been extirpated continued to show contractions of the velar sphincter when the tentacles were stimulated with particulates introduced into the water stream.

### *Peripheral conduction pathways surviving brain removal*

As noted earlier, the siphons of debrained animals continue to respond to stimulation. These responses are slow and initially local, but spread farther with repetitive, strong stimulation and may even spread as far as the other siphon and to the branchial sac, causing ciliary arrest. Spread is incremental and an order of magnitude slower than intersiphonal responses in intact animals, and the amplitude of contractions in the responding muscles is much lower. These findings were not pursued further since they essentially repeat work done on *C. productum* (Mackie and Wyeth 2000), but they serve to show that *Corella*, like *Chelyosoma*, has peripheral conduction pathways interconnecting the siphons and branchial sac that survive removal of the brain. As with *Chelyosoma*, these pathways are blocked by treatment with 10 $\mu g \cdot mL^{-1}$ of *d*-tubocurarine, showing that they are nervous.

### *Electrophysiology*

Using the semi-intact preparation described above, it was possible to evoke and record the electrical correlates of contractions in the oral and atrial siphons, and the electrical correlates of ciliary arrest in the branchial sac. The velar sphincter was selected as the site for recording from the oral siphon, because it responds locally in the context of the crossed response and together with many other oral siphon muscles during squirting. Recordings from the siphons are electromyograms and the ripples of activity seen are summed trains of action potentials, the individual spikes being very small (<20 µV). In the simplest case (Fig. 11A), gently stimulating an oral tentacle evokes a ripple of electrical events in the recording from the velar sphincter, with no detectable response in the atrial siphon. This represents the minimum observable response. In Fig. 11B, the velar sphincter again gave a short latency response, but this time, excitation spread to the atrial siphon some 200 ms after the start of the response in the velar sphincter. This presumably corresponds to the crossed response, and the ca. 200 ms delay would represent the conduction time in the afferent nerves going to the brain and efferent nerves going to the atrial siphon, plus processing time in the brain itself. In Fig. 11C, the early response seen in the velar sphincter merged into a stronger ripple of contraction exhibited in synchrony with contractions of the atrial siphon. This would correspond to a full squirt. The squirt component in the two siphons followed the initial response of the tentacular sphincter after about 200 ms, this representing conduction and processing time as noted above. In Fig. 11D, an electrode on the branchial sac recorded a ciliary arrest potential in synchrony with the squirt response of the velar sphincter. The branchial sac was left largely intact in this preparation and the electrode on the velar sphincter picked up an electrotonic echo of the massive depolarization associated with the ciliary arrest event, here seen superimposed on the ripple representing contraction of the sphincter. Finally, after extirpation of the brain (Fig. 11E), single shocks evoked a short-latency contraction of the velar sphincter, with no spread of excitation to the atrial siphon or branchial sac. The persistence of the sphincter response after debraining can be explained only by the existence of a peripheral conduction system that survives the loss of central connections, a system that includes both sensory receptors and motor pathways to the sphincter muscle. The latency and form of the response are so similar to the "minimal response" seen in animals with intact brains (Fig. 11A) that we can safely assume this short-latency response is mediated by the peripheral conduction system regardless of whether the brain is present or not.

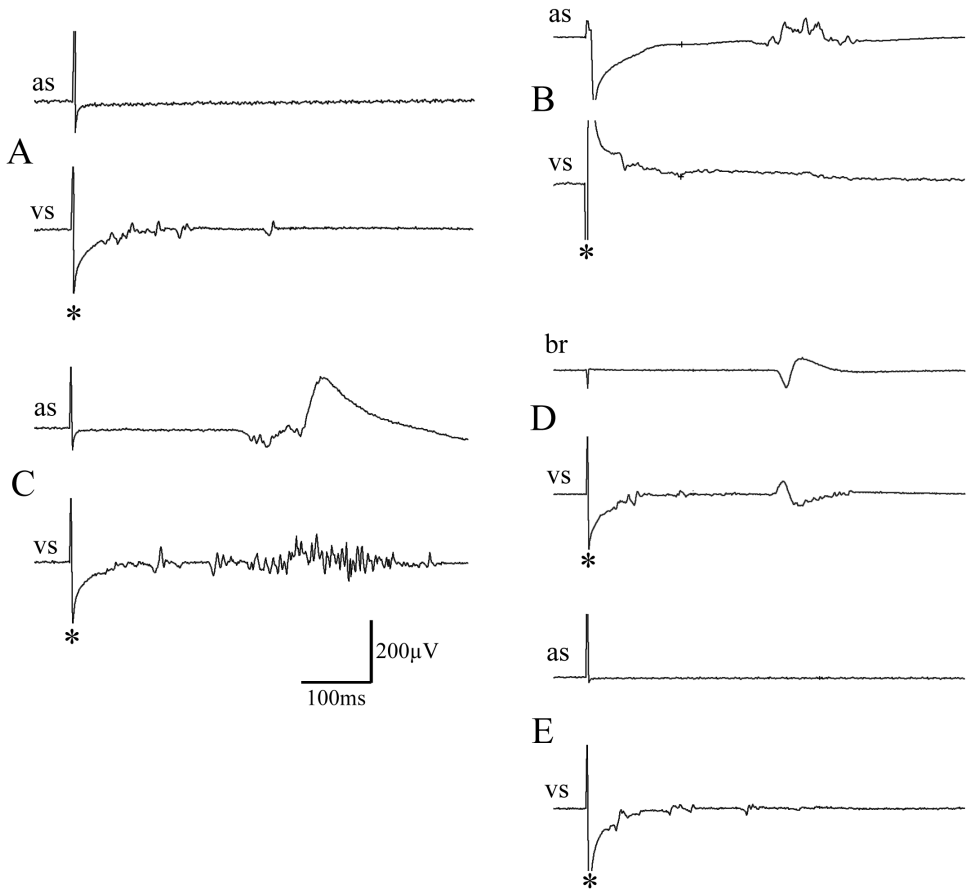
We have already noted that excitation appears to propagate throughout the whole oral siphon during crossed responses even when the velar sphincter is the only muscle in the siphon that visibly contracts. Electrophysiological recordings bear this out. Stimulation of the siphon rim or tentacles evokes a spreading response with ripples of contraction events recorded in muscles throughout the siphon, but the amplitude and duration of these events are much larger in the sphincter than in the other muscles.

## Discussion

### Primary sensory neurons in the siphons: structure and function

The presence of ciliated, primary sensory neurons in the

**Fig. 11.** Electrophysiological responses to single shocks (*) delivered to oral tentacles in semi-intact preparations as seen in Fig. 2. Several different specimens were used for these records and, while the time scale is the same in all, the voltage settings varied slightly from the value shown, which applies specifically to (C). The lower traces are electromyographs recorded from the velar sphincter (vs) in the oral siphon. The upper traces are electromyographs from the atrial siphon (as), except in (D), where the record is from the branchial sac (br), and shows ciliary arrest potentials. (A) A shock evokes contraction of the velar sphincter alone. (B) A full crossed response, with both the velar sphincter and the atrial siphon contracted. (C) A squirt. Following the initial ripple of contraction in the velar sphincter, both it and the atrial siphon contracted strongly in synchrony. (D) Arrest of the branchial cilia during a squirt. (E) After brain extirpation, the velar sphincter responded as usual to a single shock, but there was no spread of excitation to the atrial siphon or branchial sac.




siphonal epithelium of ascidians was noted by Fedele (1923) and Millar (1953), and later verified using anti-tubulin and anti-GnRH labelling (Mackie and Wyeth 2000). We have now carried out a more extensive survey of the distribution of these cells using the same methods. We estimate that there are more than 8000 sensory cells in each siphon, some located along the rim, some in the outer mantle epithelium, and some in the inner, but in all cases are present only in areas covered by the tunic. In some regions, the sensory cilia are probably inserted into pores in the tunic, as in tadpoles of *Diplosoma* Macdonald, 1859 (Torrence and Cloney 1982). They are figured thus in Fig. 7A of Mackie and Burighel (2005). Particularly round the siphon rims, the cilia appear very short and may have lost their distal tips when the tunic was removed. In other regions farther back, longer cilia are seen, which may mean that they were not inserted in the tunic.

On the inside of the siphon, the tunic extends only about one-third of the way down from the rim and this is the region where the sensory cells also lie. There is a clear line of demarcation between this zone (which we call the sensory zone) and the epithelium lining the lower two-thirds of the siphon (non-sensory zone). Not only are the two zones distinguishable by the presence or absence of primary sensory neurons, but the epidermal cells themselves differ. Those in the sensory zone have tiny cilia (ca. 1 µm long), whereas those in the adjacent non-sensory zone are unciliated. These cilia resemble the "primary cilia" that characterize many types of interphase epithelial cells in vertebrates. Such cilia were thought to be "rudimentary" or "abortive", but it appears that in many animals they are functional sensors that play a critical role in membrane signal transduction pathways (Salisbury 2004).

The line of demarcation between the two zones is also marked by the presence of what appear to be stages in the production of new sensory neurons. Rounded cells and cells with short axons are located along this line and show the same characteristic GnRH-ir as mature sensory neurons. We propose that neurogenesis in this region augments the population of siphonal sensory neurons pari passu with the expansion of the siphon as the animal grows. It is not clear, however, where the new cells come from. Production of primary sensory neurons from local epidermal cells was suggested by Bone and Ryan (1978) in the *Ciona* cupular

organ. Origin of sensory neurons by transformation of local epithelial cells in the line of demarcation remains a possibility in the present case, and the existence of primary cilia in the epithelial cells suggests that they are in a sense preadapted to become sensory neurons. Cells identified as "neuroblasts" are present in the dorsal strand of *Corella* and bear a close resemblance to the rounded GnRH-ir cells we find here; however, again there is some uncertainty about their origin, although they may be produced by delamination from the neural ectoderm of the dorsal strand (see Mackie and Burighel 2005).

Manni et al. (2006) point out that primary sensory neurons are found only in areas covered by the tunic, whereas complex sense organs (cupular, capsular, and coronal organs) lie in areas that are not covered. It seems clear that the cilia of primary sensory neurons are either inserted into the tunic, or touch it with their tips, so the tunic could in fact function as an ampulla-like accessory device that picks up and relays movements to the sensory cells.

Our experimental analysis strongly implicates the primary sensory neurons as tactile and vibration receptor units. The only other sensory component in the siphon is the coronal organ, located in the oral tentacles and velum. Truncation of the oral tentacles, which drastically reduces the size of the coronal organ, has little if any effect on sensitivity to water-borne vibrations in the frequency range of 1–200 Hz. There is no coronal organ in the atrial siphon, but we find it to be just as sensitive to vibratory stimuli as the oral siphon.

**Coronal organ structure and function**

The layout of the oral tentacles, velum, and coronal organ in *Corella* closely resembles that described in other phlebobranch ascidians, in particular another corellid, *Chelyosoma productum* (Manni et al. 2006). The sensory cells in phlebobranch coronal organs are relatively simple compared with those in stolidobranchs like *Styela* and *Botryllus*. They bear several cilia but lack the apical clusters of stereovilli, which are such a striking feature in the styelid organ. In both *Corella* and *Chelyosoma*, the sensory cells are flanked on the inner side by secretory cells and there is some evidence of an active process of secretion of proteinaceous material to the exterior. The secretory cells are not innervated, but their proximity to the coronal sensory cells suggests a functional connection as proposed by Manni et al. (2006). The epidermal cells lying between the two coronal strands on the upper side of the tentacle are ciliated. The cilia are motile and could function in transporting the secretion along the upper side of the tentacle.

Tubulin labelling shows the general layout of nerves in the tentacles. A nerve bundle runs under each row of ciliated cells and can be followed back to the velum where it merges with the general peripheral nerve plexus formed by the arborization of nerves connecting with the brain. It is not at present possible to distinguish afferent and efferent units in ascidian peripheral plexuses.

As in other ascidians examined, the coronal ciliated cells synapse with neurites at their bases. The latter typically lie in grooves formed by infolding of the cell membranes of the ciliated cells. Most of the synapses seen in *Corella* were afferents (i.e., with the synaptic vesicles on the sensory cell side), but some efferent synapses were also seen as first observed in botryllids (Burighel et al. 2003).

Our experiments show clearly that the oral tentacles detect large particles entering via the incurrent water stream, causing either crossed responses or squirting, and some degree of particle rejection. We have investigated the crossed response thoroughly for the first time since its first full description by Hecht (1918*b*). Bacq (1935) also observed the response, but Florey (1951) was unable to obtain it in *Ciona* Fleming, 1922: he states that both siphons close virtually simultaneously, or with the unstimulated siphon contracting slightly later. Clearly, he was observing squirting and not the crossed response.

The crossed response was consistently obtained in our experiments on *Corella* and we regard it as a tentative rejection response (a mini-squirt) that causes a brief interruption in the steady inflow of water, serving to eject or delay particles about to enter the siphon and possibly breaking up aggregates into smaller units. The response is graded and successive responses can sum, increasing the probability of rejection. In the example photographed in Fig. 1A, touching the oral tentacles evoked a single crossed response in which the atrial siphon contracted to less than 50% of its resting diameter. At the same time, the velar sphincter tightens (described here for the first time), bringing the tentacles closer together and making it more likely that particles will hit the tentacles rather than slip between them.

Squirting by contrast is an all-or-none response that causes violent ejection of water and contained particles from the oral siphon and atrial water from the atrial siphon. It can occur repetitively, resulting in long-term siphon closure. This occurs with exceptionally strong stimulation and is driven by nervous pacemakers (Mackie and Burighel 2005). Squirting is invariably accompanied by synchronous arrest of the stigmatal cilia in the branchial sac. Crossed responses often precede squirts, and when scoring responses to particulates the two responses were lumped together, as both clearly function in particle rejection.

Crossed responses or squirts were evoked by contact of single particles with the oral tentacles. Particles in the 500–600 μm evoked rejection responses 88% of the time, whereas those in the 355–425 μm range evoked rejection 61% of the time. Particles smaller than 125 μm evoked responses less than 8% of the time. Visual observations suggest that rejection responses typically result from sudden, direct impact of particles against the upper surfaces of the tentacles where the coronal organ is located. Large particles that enter slowly and slide down the inner wall of the siphons often get through the tentacle mesh without causing rejection responses, while similar particles carried in with the full force of the incurrent stream cause rejection on hitting the tentacles. Small particles below about 100 μm typically find their way through the mesh without striking the tentacles. If the tentacles are truncated, the animal almost completely loses its ability to reject large particles.

It is clear from these observations that the oral tentacles are mechanosensory structures. As the coronal organ is the only known sensory system present, we conclude that the ciliated cells of the organ are the mechanoreceptors responsible for triggering particle rejection responses. This does not preclude other possible functions, such as vibration re-

ception, but as removal of the tentacles does little to diminish vibration sensitivity, the coronal organ can play no more than an auxilliary role in this respect. The location of the coronal organ on the upper sides of the tentacles ensures that particles hitting the tentacles head-on or striking glancing blows as they pass through the gaps between them will stimulate the coronal organ. Whether this results in a single crossed response, summing crossed responses, or single or repeated squirts probably depends on a number of factors such as the number of impacts, their force and frequency, and the immediate past history.

It is possible that the coronal organ sensory cells are chemoreceptors, although this requires a new investigation. Nägel (1894) reported that *Ciona* and other sessile ascidians completely lack a chemical sense. Hecht (1918*b*) reported that *Ascidia atra* (= *Ascidia nigra* (Savigny, 1816)) responded to acids, bases, salts, alkaloids, and anaesthetics, but it is doubtful if any of these tests were diagnostic of a true chemical sense, as there was no way of being sure that the substances tested acted through specifically sensory pathways. Acids, bases, and salts cause general pH upset or osmotic disturbance, while strychnine, quinine, and morphine are neuroactive drugs known to affect the central nervous system directly. Thus, the evidence for chemosensitivity is inconclusive at best. Recently, however, evidence of sensitivity to injury fluids from conspecifics has been presented (Pelletier 2004), so we cannot exclude a possible chemoreceptive role for the coronal organ. It is unlikely that the primary sensory neurons in the siphonal epidermis are chemoreceptors, as they lie beneath the tunic. The sensory cells of the coronal organ by contrast are exposed to the water directly. It would be interesting to repeat Pelletier's (2004) experiments with animals from which the oral tentacles had been removed.

We have seen that the coronal sensory cells in *Corella*, as in other phlebobranchs (Manni et al. 2006), are of simpler design than those of styelids and it is unlikely therefore that the functional picture emerging for *Corella* reflects the full range of capability present within the Ascidiacea; however, our findings point to one basic function probably shared by all members of the group, that of monitoring the particulate content of the incoming water and triggering rejection responses in cases where the quantity or size of the particles threatens the efficiency of these animals as filter feeders.

**Neural pathways**

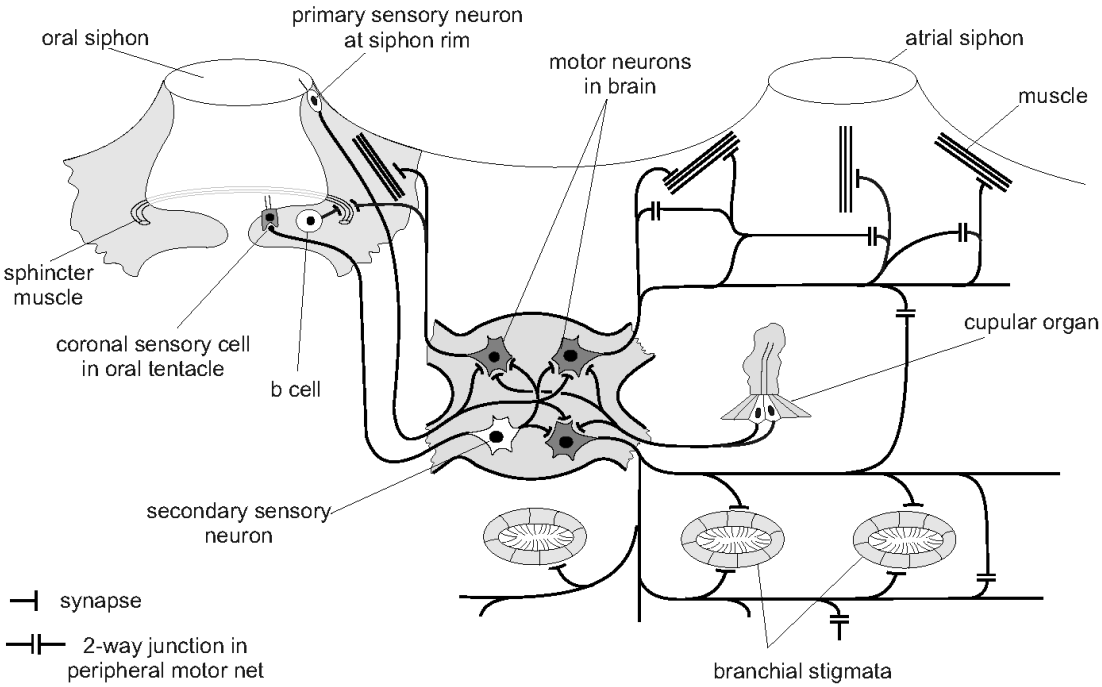
We have recorded from muscles in pinned, semi-intact preparations to investigate the pathways involved in the various responses and it has been possible to obtain records that satisfactorily represent the normal sequences of events underlying the crossed response and squirting. Stimulation of single oral tentacles was found to evoke a hierarchy of responses depending on stimulus strength: (*i*) velar sphincter contraction, obtainable with minimal stimulation; (*ii*) velar sphincter contraction followed by atrial siphon contraction, obtainable with slightly stronger stimulation and presumably equivalent to the crossed response; and (*iii*) velar sphincter contraction followed by contraction of both oral and atrial siphons, along with ciliary arrest in the branchial sac, obtainable with the strongest level of stimulation and equivalent to squirting behaviour.

Our present understanding of the neural pathways involved in ascidian behaviour is shown diagrammatically in Fig. 12, based upon a body of work summarized by Mackie and Burighel (2005). Sensory axons from peripheral sensory cells and organs (coronal, cupular) are shown as passing to the brain and synapsing with motor neurons that send their axons to the siphons and to the branchial sac. Responses *ii* and *iii* above clearly involve such reflex routes. These responses are typically rapid and vigorous and can be evoked by single, short-duration stimuli. We also have to account for conduction pathways that survive brain removal. In some cases, these remain functional for months (Mackie and Wyeth 2000). The short-latency sphincter response (response *i* above) is probably mediated by these local pathways and it persists unchanged after deganglionation. Such pathways are evidently part of a widely distributed conduction system that extends right across between the siphons and to the branchial sac, making possible the spread of contractions and ciliary arrests, given strong, repeated stimulation in debrained animals. In animals with intact brains, these purely peripheral pathways evidently serve for local responses, but the behaviour is dominated by centrally mediated reflexes. Recordings from the velar sphincter following stimulation of the oral siphon show an early (locally conducted) response followed by a later (reflexly mediated) response.

There has been considerable debate about the nature of the peripheral pathways that survive brain removal, but present evidence suggests that they are composed of axon terminals of central motor neurons interconnected synaptically (shown symbolically in Fig. 12) so as to form a peripheral motor network. (There is no histological evidence for a conventional nerve plexus, i.e., one composed of neurons with peripheral cell bodies.) The peripheral network then, by virtue of its connections with central motor neurons, will be involved in both local and reflex activities, mediating local responses on its own and delivering motor commands arriving from the brain. There are uncertainties about this interpretation that need to be resolved. For example, the local sphincter response seen in debrained animals can be elicited by the same, very delicate stimulation in the intact animal, which suggests that local sensory receptors can feed into the local motor network; a possibility noted earlier by Mackie and Wyeth (2000) in the case of *Chelyosoma* but not included in Fig. 12.

It is interesting that very similar responses are obtainable by stimulation of the siphon walls in areas where scattered primary sensory neurons are the presumed sensors, and of the oral tentacles, where the coronal organ is presumably the sensor. Input from cupular organs located in the atrial epithelium probably also cause squirting and ciliary arrests, although the physiology of these organs has not yet been investigated in such a way as to distinguish their role clearly from that of other sensors. In *Chelyosoma*, the capsular organs are vibration sensors that trigger squirting and ciliary arrest (Mackie and Singla 2002). The circuit diagram for *Corella* (Fig. 12) assumes that the cupular organ also feeds into the set of final, common motor pathways, but there could be subtle differences in the behavioural responses associated with input from the various categories of sensors that have not yet come to light.

**Fig. 12.** Circuit diagram showing the main sources of sensory input and the general pattern of innervation of the siphonal muscles and the branchial cilia. Motor neuron cell bodies are located in the brain, but two-way junctions between motor nerve terminals provide the equivalent of a peripheral nerve net. The dual innervation of the velar sphincter muscle by central motor neurons and *b* cells is indicated at the tentacle base. The diagram omits input from primary sensory neurons in the atrial siphon, which mirrors that shown for the oral siphon. Modified and redrawn from Fig. 15 in Mackie and Burighel (2005).




In contrast to the primary sensory neurons, each of which sends an axon to the brain, there is a high degree of convergence in the afferent pathway from the coronal organ (>10:1). Convergence was also noted in *Botryllus* (Burighel et al. 2003). It appears that the coronal organ functions largely on a quantitative basis, the strength of the afferent signal varying with the number of sensory cells stimulated, and that this information can be carried effectively by relative few afferent neurons, making 1:1 transmission to the brain unnecessary.

The role of the mysterious *b* cells cannot be satisfactorily explained at the present time. They have not been described previously and present novel (and puzzling) features. Firstly, they do not appear to be connected either to the nerves associated with the coronal organ or to the general motor network. Secondly, their processes clearly run out along the sphincter muscles, which suggests that they are motor axons that excite the sphincter, providing this muscle with a motor innervation supplementing input from the main motor net. If the cells are motor neurons, then they are highly unusual because all other known motor neurons in ascidians have their cell bodies in the brain. Thirdly, the *b* cells resemble primary sensory neurons in having peripheral cell bodies, being grouped in pairs, and each having a single axon; however, unlike primary sensory neurons elsewhere, they lack cilia or any other obvious sensory specializations.

In *Polyandrocarpa misakiensis* Watanabe and Tokioka, 1972, a new sensory structure has recently come to light in a location similar to that of the *b* cells in the two species of *Corella* described here (H. Koyama, personal communication). In *P. misakiensis*, the tunic extends down inside the oral siphon to the bases of the tentacles where it forms a bulge. A group of primary sensory cells lying in the underlying epithelium project their cilia into the tunic bulge. The presence of indisputable primary sensory neurons at the tentacle bases in *P. misakiensis* is consistent with the view that the *b* cells of *Corella* originated from such cells which lost their cilia and became subepithelial. Thus, the simplest, if unorthodox, explanation for the *b* cells would be that they are sensory cells whose axons directly innervate the sphincter muscle. Sensory motor cells are known in hydra, *Aiptasia pallida* (Agassiz in Verrill, 1864), and other cnidarians (Westfall et al. 2002). Possibly the *b* cells function as proprioceptors that amplify motor events initiated by the main motor network — this might account for the ability of the sphincter muscle to contract when other muscles in the oral siphon fail to do so, even when we know that excitation has propagated throughout the whole siphon. Alternatively, they might be sensors tuned to detect small fluid displacements or pressure changes transmitted through the blood as their cell bodies are suspended in the tentacular blood sinus. The arrangement might be similar to that proposed for certain crustacean stretch receptors, where compression of one part of the cell is reflected in swelling at another, resulting in sensory transduction (Wilson and Paul 1990). It would be premature to speculate further about these highly enigmatic cells, which are unlike anything previously described in the Tunicata.

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