Entrainment of Chaotic Oscillations in a Colonial Tunicate.

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Research Article

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Abstract

Background

Tunicates comprise an invertebrate, chordate subphylum which has been shown to be the closest group to vertebrates. Colonial tunicates are clusters of genetic clones generated asexually from a single free swimming larval “tadpole”. Each individual, or zooid, of the colony has a peristaltic heart which circulates blood through that individual. In addition, each zooid is connected to a common, external vascular network. This vascular network has radial extensions that end at the colony periphery in bulbs, or ampullae, which contract and expand to generate reciprocating flow between ampullae and zooids. Surgically detached ampullae continue to beat.

Results

Quantitative scans of videos of individual ampullae in a young *Botrylloides viocella* colony demonstrate ampullae contractions are often in phase, with occasional abrupt phase shifts out of and back to synchrony. The vessels connecting the ampullae to the zooid also contract, mostly in phase with the ampullae. Total volumes pumped by this colonial system are a significant fraction of the zooid volume, since it contracts 180 degrees out of phase and at the same frequency as the ampullae. Reversals of the peristaltic heart are at least partially synchronized with ampullae contractions. Ampullae that have been surgically detached from the colony contract at a more uniform rate with more symmetrical profiles than when part of the colony.

Conclusion

Contractions of the ampullae and associated vessels pump sufficient blood in and out of the zooid that they should be considered functional hearts, and the partial synchrony of ampullae contractions results in a larger blood flow compared to an alternative asynchronous contraction pattern. The manner in which the ampullae abruptly fall out of and back to synchrony indicates synchrony is due to entrainment while the out of phase contractions of the zooid may be a direct result of pumping. The shape of contraction curves of detached ampullae pairs is almost indistinguishable from a pure sine wave, indicating that the more complex original pattern was due to interactions between out of phase ampullae. Ampullae and associated vessels might be analogous with the system of lymphatic vessels in vertebrates.

Background

Ascidian colonial tunicates contain two distinct but connected blood compartments. In each zooid blood is circulated by a peristaltic heart. This circulation system is connected to a common or colonial network. Typically two vessels extend from each zooid radially to a vessel ring and extensions from the ring extend to terminate in bulbs or ampullae, approximately evenly distributed around the edge of the colony. These ampullae expand and contract about every 100 seconds, mostly in synchrony. Bancroft, more than 100 years ago [1], observed blood flow and contraction in groups of ampullae surgically removed from

colonies. Thus while ampullae and peristaltic heart are connected, they are not dependent on each other to pump blood.

Botrylloides viocella is the tunicate species studied in this report. The very similar *Botryllus schlosseri* is the subject of many more publications. The major difference between the species is the pattern of zooid clustering. Large colonies of *B. viocella* contain long rows of zooids, with each zooid having an exposed oral siphon but expelling water from large common openings that appear in apparently random locations in the colonial tunic. In *B. schlosseri* colonies are made up of many star-like groups of up to 12 zooids, again each having an exposed oral siphon but sharing a common cloacal opening at the center of the cluster. The number of ampullae in colonies immediately after the founding “tadpole” has attached to a substrate varies between species, in *B. viocella* there are between 30 to 40.

The present study is the first comprehensive quantitative description of the sizes and shapes of components of the colony recorded as a function of time. However, this measurements are of use only if they add to understanding the biology of the tunicate. The recent review “The biology of the extracorporeal vasculature of *Botryllus schlosseri* “ [2] is thus very relavent. The major immune role of the vasculature has been reviewed by Rosental et al. [3], ontology of the anatomy and development of B. Schlosseri is described in [4], culture and reproduction is reviewed by Gasparini et al. [5], angiogenesis of the vasculature is reviewed by Tiozzo et al. [6], and the review by Manni et al. [7] is also very useful.

Methods

Collection and maintenance of tunicates

Tunicate colonies were collected from the sides of floating docks in Sausalito, California and transferred to an aerated aquarium. Tadpoles released from these colonies during the next 24 to 48 hours were collected by pipette and transferred to glass cover slips in about 0.5 mL of SW. When tadpoles had attached the dish was flooded with SW which was then changed daily. In some cases colonies that had triggered to the water surface were gently rotated and pressed to the glass surface. To increase contrast colonies were stained for 2 minutes with a 1:10 dilution of a 10 percent solution of Neutral Red and then washed 3 times in SW.

Microscopy and image collection

Growing colonies were observed using an Olympus SZX16 stereo microscope, typically with a 1x objective and a zoom settings of 1 to 4. Darkfield illumination from below was provided by an integrated LED ring. Images and video were recorded by an attached Canon Rebel 600D camera.

Image analysis software

Color MOV files were converted to greyscale image sequences with the Apple Quicktime Player 7 application. These images were scanned using a custom Java plugin for the NIH open source application ImageJ. The scanning strategy is a hybrid between human definition of the object to be scanned and the

programmatic scanning of that object. Thus the first step is to make a black mask, i.e. a solid black blob, that just fills the object to be scanned, e.g. one ampullae. The commercial software PhotoShop was used to make this mask. This mask image is then inserted as the first frame in the image sequence to be scanned and it directs and defines scans of the remaining images.

The user picks a direction for the scan lines and a threshold to define the ends of chords across the target. The total number of pixels in all chords is the area. The ends of chords found during scanning are displayed superimposed on the actual image to enable the user to adjust thresholds etc. to obtain a good value for area, or in the extreme to just abandon the attempt to scan the image. The ability to curate the scanning process is essential to produce reliable data.

Heartbeats of the peristaltic heart were defined by periodic variations in grey levels over a small, e.g. 16 pixel, mask.

Analysis and graphical presentations were accomplished using commercial software, e.g. Mathematica.

Results

The free swimming larval form, or “tadpole”, of the tunicate B. *viocella* is released from its colony and swims for 1 to 3 days before attaching to a solid surface where it metamorphoses to the adult form as a filter feeder in approximately 24 hours. The larvae is large and complex compared to most other tunicates, and a heartbeat can be seen. The circulatory system is highly compressed at this stage and is further obscured by the tunic gel enclosing the tadpole. However, the approximately 30 ellipsoidal ampullae are visible as a parallel bundle around the surface of the anterior. The ends of these ampullae will be the major regions of attachment to the solid substrate, after which the ampullae rapidly spread outward radially on the surface.

Even at the time of attachment several small buds can be seen on the surface of the tadpole. Each bud is developing into a new individual, or zooid, to augment the parental zooid in the growing colony. The time chosen in this report to document pulsations of the ampullae and other parts of the colony is a compromise between delaying long enough for the ampullae to be clearly visible as they spread out from under the zooid body, but before the zooid, buds, and the associated circulatory network grow and become even more complex. The exact number of ampullae, the pattern they form on the surface, the details of the connecting vessels, the size and number of zooid buds, and the periods of contractions vary from colony to colony. The first zooid of the colony is sometimes called the oozooid, to emphasize that it has developed directly from the egg. However, only young colonies, which contain only a oozooid are considered here and thus the distinction will be dropped.

General conclusions made in this report have been distilled form observation of more than 100 colonies over two years. However, the majority of actual measurements presented in the Figures come from one colony that had a combination of characteristics that facilitated observation and quantitative measurement. The focus on one colony reveals how all its parts function together.

A typical tunicate colony

The colony seen in Fig. 1 was initiated 4 days earlier by a 1 day old tadpole. This grey level image was derived from one frame of a color video sequence of 2048 seconds. The area of ampullae number 1 was obtained by a computer controlled scan documented in the insert and described in detail in in the Methods section. The solid white lines in the insert are the ends of chords that define the area.

The vessel network connecting ampullae and zooid

The vessel network that links the ampullae and zooid can only be partially discerned in Fig. 1. In order to obtain a more detailed and functional description the contrast was greatly increased using software and blood cell movement was visualized by repeated examination of accelerated (time-lapse) segments of the video.

The resulting map is presented in Fig. 2. as a net of red lines that for the most part overlay the sometimes faint grey lines seen in Fig 1. There are also small ampullae that may grow into larger ones, but their behavior is not described in this report.

The basic topology of the net is a set of radial vessels from ampullae to a vessel ring which appears here to be connected to the zooid body at only 5 locations, but here the central segment of the network is obscured by blood movement in the zooid body. Previous observations indicated that typically these vessels connect to circulation in the zooid or associated buds at 2-4 points. Note that a few ampullae are almost directly connected to each other, e.g. 20 and 21, while others are far more isolated, e.g. 15 and 16.

Contractions of one ampullae

Contractions of ampullae diameters along the tip-base axis are generally in phase, which was the case for all the ampullae that could be reliably scanned in the colony seen in Figure 1. However, for some ampullae in some colonies, contraction of some segments, a few percent of the total, can be significantly out of phase. Thus the mechanism of contraction does not absolutely ensure synchrony.

As seen in Figs. 1 and 2 the two dimensional profiles of most ampullae appear to approximate an ellipse with a length about 2-4 times longer than the width. Observation of accelerated videos suggested that width changes more than length during the rhythmic pulsations and measurement confirms this conclusion.

Actual measurements of changes in width and length of amp 1 over a period of 1024 seconds are seen in Fig. 4. Though the ampullae's width is only about 1/3 its length, the width changes by about twice the absolute number of pixels. Changes in the widths of ampullae 1, 19, and 25 were found to have a range of 37, 27, and 23 percent, while the lengths had a range of 7.5, 5.3, and 8.7 percent respectively. Thus rather than being similar to elastic balloons that expand and contract symmetrically, ampullae are more similar to elastic cylinders that expand and contract mostly in radius. One consequence of this

asymmetry is that the measured changes in area of the profiles are fair approximations to changes in the surface area of the ampullae.

Fig. 5 is a plot of the contraction profile of ampullae 1 for 2048 seconds and a sine wave picked by eye to match the regular parts of the ampullae curve as best as possible. This sin wave has 25 cycles in 2048 seconds, thus a period of 82 seconds. The average period of ampullae contractions in 12 other colonies was found to vary from 62 to 143 seconds, with a mean of 104 seconds, thus the period of the colony seen in Fig. 1 is fairly typical. While the area profile of ampullae 1 appears similar to the sine wave in many segments (that is how this sine wave was chosen) in the ampullae profile differences between peak times are not constant, peak shapes change, and peak extremes are not constant.

An intuitive way to characterize an oscillating curve is to measure the times between the maxima, the peak to peak times. The average peak to peak time for ampullae 1 is 76 seconds, quite close to the constant 82 second peak to peak time of the sine wave, but the standard deviation of the ampullae times is 22 percent, because most ampullae peaks often come slightly before or after the peaks of the sine wave.

A more complete comparison between the contractions and sine waves can be obtained by computing the Fourier power spectrum (FPS), seen in Fig. 6, which is sensitive to peak times, heights, and shapes. The major peak for ampullae 1, at a frequency of 26 per 2048 seconds (period of 79 seconds) is only 15.9 percent of its total power, with the remaining distributed over many higher and lower frequencies. As expected (or defined) the power spectrum of the sine wave with that frequency has but a single peak containing 100 percent of the power at this frequency.

Contractions of a group of ampullae

To see variation and search for patterns of similarity between contractions of many ampullae with respect to both position and time we need to be able to see a lot of data in one graphic, but not with great precision. Coding ampullae areas as colors makes this possible. The areas of 15 adjacent ampullae, more than half the colony perimeter, over a time span of 2048 seconds are seen in Fig. 7 as 15 parallel vertical time lanes, with yellow representing large, green average, and blue small areas.

Ampullae 14 and 15 have similar patterns. Ampullae 20 and 21are seen to share many dramatic blue bands, for example at 200, 1050, 1200, 1570, 1800 and 1950 seconds. This is not surprising since each of these pairs are neighbors connected by a short vessel segments as seen in Fig. 2. Ampullae 16, 17, and 18 have similar large peaks for the first 800 seconds, but share some, but not all, blue bands after that. Lanes 9 and 10 share many bands. In general neighboring ampullae tend to be similar, and distant ones different.

However, the lack of a simple or even symmetric relation between contractions of most ampullae is consistent with the complex and mostly non-symmetric geometry of the vessel net connecting the ampullae with each other and the zooid. It is interesting that adjacent segments of distinct peak patterns

do not seem to “diffuse” laterally to neighboring ampullae, they just come and go in their own neighborhood.

The periodicity of each of the 15 ampullae can be assessed in Fig. 8, a color map of their Fourier power spectra. The total power in the spectra displayed in this map have not been normalized for each ampullae, thus ampullae that have more extreme contractions have higher total values over all the frequencies. This is illustrated by more red in lane 21 compared to lane 9. The considerable differences between ampullae are clear.

Correlations between pairs of ampullae

Only an intuitive feeling for correlations of contractions between the 15 ampullae can be obtained by examination of Figs. 7 and 8. In contrast the actual values of all the possible 105 pair wise correlations of contractions are represented as colors in Fig. 9.

Three red 2x2 squares along the diameter of Fig. 9 indicate the high correlations between the ampulla 10-11 pair, the 14-15 pair, and the 20-21 pair. In contrast, the blue rectangles toward the upper right and lower left corners represent the high negative correlation between the 20-21 pair with ampullae 8 through 12. Ampullae that are neighbors are more likely to be correlated than pairs that are distant, but the detailed pattern is complex and any other generalization is not obvious.

Rolling time correlations between ampullae

A rolling time or time windowed correlation between two sequences is a series of correlations between two small subsets of the sequences that moves along in time. It thus reveals transient time dependent correlations that are averaged out when the entire series are compared.

It is seen in Fig. 10 that the two ampullae are highly correlated in a 60 second window for the first 900 seconds, then become much less for 100 seconds, then return to high correlation for another 500 seconds. The remainder of the time they abruptly go into and out of correlation several times. A sequence of high and low correlation periods is characteristic of two oscillators that are partially entrained.

Phase portraits of two ampullae

The phase portrait, showing the trajectory of an objects motion, or behavior, in phase space, is a common tool used to understand dynamical systems. It can reveal subtle aspects of a system and is not linked to a specific function, as Fourier analysis is. The top panel of Fig. 11 shows the plot of area versus time for ampullae 9, with the brown segment indicating the segment to be plotted as a phase portrait in the lower panel. The curve displayed there, starting with the green and ending with the red segment, is the ampullae area versus the time derivative of that area. If the time axis extends upward from the paper, this curve is a helical trajectory collapsed on the page.

The trajectory for a pure sine wave would be a series of superimposed ellipses, one for each complete cycle of the wave. If the area of the ampullae was suddenly altered by an external force, but the system was stable, the trajectory would gradually return to the original elliptical curve, which is thus called an attractor. In contrast, the trajectory for ampullae 9 is a series of loops of increasing size reflecting the increasing size of the cycles in the top panel. The trajectory is flat on the left (most negative values of area) because the derivative changes a great deal over a small range of area, the actual data curves are sharper on the bottom. A model for ampullae 9 would be an oscillator with an intrinsic frequency being influenced by modest, gradually increasing external forces causing excursions from the intrinsic ellipse.

The trajectory for ampullae 21 over the same time period, seen in the bottom panel of Fig. 12, is quite different, a reflection the different pattern of the raw date seen in the upper panel. Three of the trajectory loops have small subloops or extrusions. These correspond to small inflections or peaks in the data. Thus strong but transient forces are modulating the behavior of this ampullae.

Vessels connecting ampullae also contract.

Observation of time lapse versions of videos of colonies revealed that the widths of all vessels connecting the ampullae and zooid body were contracting along with ampullae contractions. A scan of a vessel near an ampullae, seen in Fig. 14, confirmed and measured these contractions. In fact the percent change in the vessel width is perhaps slightly larger than the change in ampullae area for this specific region.

The sum effect of all ampullae in the colony

Since contractions in most ampullae are not in complete synchrony, the sum effect of these contractions, pumping blood in and out of the zooid body, would be expected to be less than maximal because some flow will just be from one ampullae to another. It was possible to scan 19 of the 27, ampullae, with good sampling around the periphery of the colony. The sum of all 19 was a curve very similar to that seen previously for individual ampullae, with a range of 19.1 percent from the mean. However, the mean of the range of individual ampullae was 30.5 percent. Thus lack of complete synchronization has reduced net pumping to 63 percent of the possible maximum.

The zooid body and its peristaltic heart

In preceding sections it has been shown that contractions of the ampullae and associated vessels are sufficiently synchronous to produce a net flow in and out of the zooid body with a period of about 80 seconds for this colony. This flow must cause parts of the zooid to swell and contract with the same period but opposite phase. However, the zooid is not a solid mass of tissue, rather it can be approximated as a small posterior region containing viscera plus two large concentric cylinders enclosing sea water. The outer cylinder is the body wall and the inner cylinder the brachial basket that supports the mucus feeding net. Thus, while some portion of the body must expand and contract, all that is clearly visible in the videos is the outer profile of the zooid.

However, measurement of the area of this outer profile shows that in fact it does contract rhythmically with the same frequency but mostly 180 degrees out of phase with ampullae contractions, as seen in Fig 14.

A peristaltic heart in the posterior region of the zooid body, with a beat rate approximately 100 times faster than contractions of the ampullae, circulates blood throughout the zooid. This heart periodically changes direction, which can be identified, in favorable cases, by viewing videos of this colony. In Fig. 15 the direction of blood flow generated by the peristaltic heart is represented by the rectangular curve alternating between 100 percent in the anterior direction along the endostyle, and 100 percent to the posterior. Contractions of the ampullae are superimposed for comparison. Starting at the left end of the graph it is possible to associate a minimum of the ampullae curve with a heart flow transition across most of the graph. Thus, in this colony, flow due to the peristaltic heart appears to be entrained with flow due to ampullae contractions, but with twice the period.

Contractions of a detached pair of ampullae.

In 1899 Frank W. Bancroft published observations on contractions of ampullae of colonial tunicates in an report[1] that is quite accessible today. He also described contractions in groups of ampullae that had been surgically separated from colonies.

Fig. 16 describes the contractions of the most simple group, a pair. These detached ampullae were connected to each other by a short vessel segment, similar to that seen between ampullae 20 and 21 in Fig. 2, however they came from a different colony. The uniformity and symmetry in these pair of contractions is apparent to the eye. The average of the peak to peak times was 127 seconds, with a standard deviation of 9.4 percent. The curves are almost indistinguishable from sine waves, with 94 percent of the total power spectrum in the main frequency.

Contractions of single detached ampullae

Even single detached ampullae contract with periods similar to those observed when they were attached to a colony. While the total volume can’t change in one isolated ampullae because blood is incompressible, the ampullae can and does oscillate as can be seen by blood cell movement and by changes in shape as documented in Fig 17.

In all ampullae described in this report changes in shape or size were correlated with movement of blood cells. If ampullae are part of a colony blood cells move in or out of the ampullae, in a detached pair blood cells move from one ampullae to the other; in detached single ampullae blood cells move from one end to the other. Blood cell movement is usually the only way to see changes in shape or size when viewing in real time with a microscope because changes in shape and size are too slow for the human to perceive directly.

The image of a single detached ampullae, the fifth in a series of 6, is seen in the upper left panel of Fig. 17. A segment of this and a another ampullae in the series were scanned and the results are presented in

the middle panel. Note that there is nothing fundamental about this segment, it was chosen merely because it changed the most, other ampullae change shapes in different patterns.

The time course of contractions of the series of 6 were followed for approximately 1000 seconds. In contrast to previous observations, the results of these scans are not areas of ampullae, but just surrogates to follow oscillation. Thus, only the times of peaks have obvious meaning. The mean periods and standard deviations of peak-peak times are seen in Table 1 and the curves for ampullae 1 and 5 are presented in the lower panel of Fig. 17.

Table 1. The means and standard deviations of peak to peak times for 6 single detached ampullae.

Ampullae	mean (sec)	stan dev (pc)
1	68.2	6.4
2	67.3	5.0
3	65.9	6.3
4	68.6	7.9
5	68.0	4.0
6	68.4	3.7

The first and fifth ampullae seem so similar as to invite a comparison. Thus, the first peak of each was aligned and the two plotted together to make the bottom panel of Fig. 17. While the peak heights of each of the two ampullae change with time and pattern, the peak times match up to an amazing extent, with the 14^{th} peak of both matching within 3 seconds out of the total of almost 900.

Discussion

Summary of results

This report presents area versus time profiles from images of different parts of the colonial tunicate *B. viocella* and documents their rhythmical contractions. In the first section a single colony is observed over a period of 2048 seconds. The ampullae contract and expand, mostly in synchrony with each other and with contractions of the network of smaller ampullae and vessels connecting them to the zooid. These results are consistent with the qualitative observations made by Mukai et al. [8].

Fourier power spectra of different ampullae have a variable fraction, but always less than half, in the main colonial frequency. Phase portraits of contractions of some ampullae are almost circular, consistent with sinusoidal contractions, while portraits of others show complex chaotic behavior. Rolling correlation plots of neighboring ampullae pairs show that they transition from periods of being highly correlated to quite uncorrelated and back. Thus correlations over the full 2048 second period are a time average of

being in and out of synchrony, and not a constant intermediate value. This pattern is characteristic of entrainments.

The sum of contraction profiles of half of the ampullae of the colony is a semi-sinusoidal curve train with a range about half as large as individual ampullae, and thus blood must flow reciprocally in and out of the zooid body. The body is not a solid mass of tissue, but mostly cylindrical space containing sea water, thus it need not change its external shape due to this flow. However, it does contract and expand, 180 degrees out of phase with the ampullae.

Blood is circulated throughout the body by a peristaltic heart which reverses direction about every 100 seconds. In the tunicate studied here these heart reversals are mostly correlated with ampullae contraction in a 1:2 pattern, i.e. when the ampullae contractions are maximal the heart changes direction, and thus the two appear to be entrained.

It has been known for more than a hundred years that groups of ampullae surgically detached from the colony continue to contract [1]. Thus contraction is an intrinsic activity of ampullae. In the present study pairs of detached ampullae connected by short vessels have been observed to contract with much greater regularity than the ampullae in colonies. The time profile of the pairs is very close to a sine wave, with the members of the pair exactly 180 degrees out of phase. In this case it does not seem appropriate to call the process entrainment, it is more like a hydraulically coupled reciprocating engine in which the two pistons are linked but 180 degrees of phase. The resulting symmetry could be partially responsible for the extreme regularity of the flow.

Single detached ampullae also contract, with a periods similar to those when they were part of a colony. However, the shapes which make up these contractions are very different than seen in connected ampullae, because the volume of each ampullae must remain constant. Thus only the times between peak values are compared to connected ampullae. Periods of six neighboring detached ampullae were found to be remarkable similar, which could be the result of entrainment by forces or electrical fields unrelated to blood flow.

It is proposed that the somewhat irregular contraction patterns of ampullae in a colony is due to interactions with neighbors with very similar but not identical periods. Since these are independent oscillators which fall in and out of synchrony with their neighbors, it is an example of entrainment [9].

Entrainment

Entrainment is the synchronization of independent oscillating or rotating units by exchanges of modest energy compared to the total energy of the system [10, 11]. Entrainment is very different from a cause and effect relationship such as the engine and propeller of a ship, and quantitatively different from an effectively deterministic phase lock that can involve large amounts of energy, as seen for example between generators on a common electrical grid.

Ampullae neighbors tend to have contraction profiles that are similar while those more separated, for example on opposite sides of the colony, have more uncorrelated profiles. Ampullae connected by short vessel segments tend to have more correlated contractions than those connected by longer segments. Thus the highly visible hydraulic connection between ampullae might seem to be the major linkage between them.

However, while in the microscope ampullae look like pods, and vessels look like tubes, in reality they are ellipsoidal and cylindrical cavities in the tunic, coated on the inside by a thin layer of epithelial cells. Thus, when these ampullae and vessels contract, the tunic they are embedded in must contract, and the mechanical strain is presumably transmitted to all parts of the colony in proportion to proximity.

In addition, contraction is presumably powered by actin or actin-myosin contraction, and these molecular processes must be synchronized among the cells of each ampullae. This synchronization is likely to involve waves of electrical depolarization moving across walls of the ampullae, mediated by gap junctions between cells, and might well bleed over to neighboring ampullae. Thus, both mechanical strain and electrical fields could be part of the entraining mechanism.

Descriptions of vessels and the peristaltic heart are complicated

It is clear that vessels contract to approximately the same extent, expressed in percent, and are in approximate synchrony with the ampullae. However, measurement of internal vessel diameters is subject to greater error compared to interior ampullae diameters because the sometimes irregular vessel walls are thick compared to their internal diameter and the small dimensions are sensitive to focus, vibration, and other observational artifacts. A more fundamental problem is that vessels are clearly not a single unit, but rather an extended bifurcated net, with a major fraction at least partially obscured by the zooid body. Since ampullae sometimes fall out of synchrony with each other, at least some segments the vessels that connect them must also, but how is this asynchrony distributed along the vessel net? A detailed description and analysis of a system this complex is beyond the scope of the present investigation.

Reversals of the peristaltic heart can easily be plotted against the average ampullae contraction cycle, as was done in Fig. 17. However, simultaneously obtaining good data on ampullae and the heart, imbedded in the middle of the zooid body, requires a specific alignment of the focal plane and usually a willingness to compromise the view of many of the ampullae. Since this study focused on ampullae, limited data on the heart was obtained. Some observations, not presented in this report, indicates that heart reversals can be entrained 1:1 with ampullae contractions, in addition to the 1:2 pattern described in Fig. 17. Since the energy exchanged between entrained oscillators can be small, it is not uncommon to have different integer ratios between periods under different conditions.

Does entrainment benefit the tunicate?

Ampullae and attached vessels are made of cells, and cells need glucose for energy and substrates for biosynthesis and repair, e.g. amino acids. The only source for these nutrients are the stomach and

intestine in the body of the tunicate, and these nutrients must be transported to the ampullae by blood flow. The peristaltic heart that distributes blood efficiently by circulation through the body has little influence on the reciprocal blood flow in and out of the ampullae. Instead, ampullae must rely on the reciprocal flow which their contractions provide. If contractions of the ampullae are completely uncorrelated with each other, the net pumping effect would be much less than if they were completely synchronized, and much of the fluid flow would be between ampullae and not between the ampullae and the zooid body.

Some of the previous Figs. show contractions profiles of different ampullae that are essentially identical for many contraction cycles, while others are very different for at least several cycles. The mean of the ranges of projected areas of the 15 individual ampullae described in Figs. 7-9 for the 2048 second time period are 31 percent, while the range of the sum of the 15 ampullae during that same time period is 17 percent. Thus, as expected, the range for the sum is lower than expected if synchronization were complete. Only half of the ampullae were analyzed in the Figs. mentioned, and the difference between the observed range of the sum and individuals would be expected to be larger if all ampullae were followed.

The predicted decrease in range for completely uncorrelated contractions depends on the model used. If each of 15 mock ampullae had sinusoidal contraction curves of the same amplitude and frequency, and ranged between +1 and -1, and were completely synchronized, the sum would range between -15 and +15, for a total range of 30. If instead each of the 15 had a random phase difference from its neighbors, the mean range in a simulation with 1000 different combinations of random phase differences had a total range of 6.7, about 4.5 fold lower than completely synchronized. This would suggest that the tunicate achieves about half the possible benefit of complete synchrony.

The out of phase contraction of the zooid body is not surprising, blood expelled from the ampullae has to cause some part of the zooid body to expand, thus it may be more cause and effect than entrainment. It's impossible to determine the fraction or even the sign of the work of pumping done by the body just by observation; active body contraction may not occur, and if it does it could oppose or enhance ampullae contraction. The outer body wall of tunicates certainly possesses muscle, and tunicates often expel a large fraction of the enclosed water when an irritating object is drawn into the feeding basket; they are sea squirts.

The detailed mechanism of entrainment of ampullae with reversals of the peristaltic heart is certainly not obvious. However, one would guess that when ampullae contractions pump blood into the zooid body it creates a higher pressure at one end of the peristaltic heart tube than the other.

Relation to observation and theory of coupled oscillators.

In this report the tunicate colony is described as a dynamic system, a collection of components which oscillate with time and interact with each other. The study of dynamic systems, especially those with non-linear behavior, is a major field of applied mathematics and in that field there is great interest in coupled oscillators. A history of development of the theory of these systems to the year 2000 was recently

published by Strogatz [12]. Reviews of the study of increasingly complex networks have been made by Rodrigues et al. [13] and phase analysis techniques by Pietras and Daffertshofer [14].

Theoretical studies typically start with a set of differential equations and investigate how the system they describe proceeds from an initial starting position to a stable point, limit cycle or chaotic behavior. The parameters of the equations are then varied to produce maps that reveal the regions of these behaviors. However, here we don't have formulas to understand but an animal to observe, and we can't start the animal at different states or change its parameters, (see last sentence of this paragraph). However, a reasonable start is a quantitative description and analysis of the steady state, which was the goal of the present work. The correlation matrix of ampullae to ampullae motion, Fig. 9, is certainly one measure of the interactions between oscillators. However, it's not at all obvious that the observation time of 2048 seconds is long enough to reveal the representative structure of these oscillators, e.g. in the first lane of Fig. 7 describing ampullae 8 there is a series of peaks with high values in the first 600 seconds, but no similar behavior in the remaining 1500; what is the average pattern? It's natural to just request a much longer data set. However, growth and change in colony morphology occurs in a similar time span, e.g. ampullae that are spaced sufficiently apart to admit good scans will often not be after 60 minutes.

Variability of anatomy in this colonial tunicate is much larger than seen in a typical vertebrate. At the stage of a mature tadpole, variability may be comparable, but development after attachment is dependent on many factors, especially the geometry of the surface. For example, in many cases the tunicate "attaches" to the water surface, remaining inverted while ampullae spread radially. The animal may then fall from the surface and adhere to a solid substrate by a few ampullae, its shape a very distorted version of the symmetrical colonies favored by a scientist intent on observing as many ampullae as possible in a structure as symmetrical as possible. Even among colonies that are reasonably symmetric, the topology of the colonial vessels is highly variable and changes hourly. For this reason that it most useful to choose one colony most amenable to study and measure its activity as completely as possible over an hour or two.

Homologous structures in vertebrates

The walls of ampullae and colonial vessels don't seem similar to either vertebrate skeletal or cardiac muscle. This leaves smooth muscle, a tissue type so varied that an entire chapter in a recent two volume series on muscle is named: "Heterogeneity of Smooth Muscle" [15]. In addition, essentially all of the structures in vertebrates that contain smooth muscle have been observed to contract spontaneously under certain conditions [16].

The structure and function of ampullae and the associated vessel net suggest homology to the lymphatic system in vertebrates. Like the ampullae the lymphatic vascular tree is linear as opposed to circular, in that the ends of the branches are closed, the interstitial fluid seeping in between slits between the cells of the vessel ends. Intrinsic contractile activity of the lymphatic vessels is an important, if not the only, mechanism driving lymph flow [17]. However, unlike the tunicate system, contractions in the lymphatics are peristaltic, flow is unidirectional, and is facilitated by valves. The blood cells in ampullae play an

important role in the immune rejection of non-homologous tunicate colonies contacting the edge of the colony. The lymphatic vessel system in vertebrates also plays an important role in transport of immune cells However, vertebrate lymph has a composition distinct from the blood flowing through the heart, since the majority of blood cells, the oxygen carrying erythrocytes, do not leave the capillaries, and the protein composition us similar to the interstitial fluid. However, this may not be a meaningful distinction here since tunicates blood cells don't carry oxygen and there is no interstitial fluid.

Instead the ampullae system could be seen as homologous to the arterioles of vertebrates. This vessel system does not usually contract rhythmically but does have a "myogenic response" to an increase in blood pressure in which the vessel diameter initially increases, but in the next approximately one hundred seconds smooth muscle cells contract to reduce the diameter to a value smaller than that before the pressure increase. A response of this type could be a derived activity of the contraction system of ampullae. There are other vascular system in vertebrates that share some features with the colonial system, e.g. the myoepithelial cells in the ducts of the mammary gland [18].

Models for ampullae contraction

Since detached single ampullae contract autonomously one might hope to develop a simple model for contraction that would describe the time profile and period in term of a few known components, perhaps of smooth muscle, using the stretch activated T-type voltage-dependent Ca^{++2} channels [19]. Medium sized bio-molecules, such as ATP or acetylcholine, with molecular weights of about 500 Daltons have characteristic diffusion times of 100 seconds over 300 μm, the approximate size of ampullae, and thus could play a role in any model.

The detached ampullae pair has a seductively simple, repetitive, and symmetrical contraction profile that is almost indistinguishable from a sine wave, and thus might seem a useful start. However, this symmetry could be misleading, since by the topology of the pair the negative part of the cycle for one member must be the positive part of the cycle for the other.

To make a model it might seem helpful to have the contraction profile when the blood pressure was constant, e.g. when the ampullae was connected to a large reservoir which was at constant pressure. However, all contraction profiles presented in this study are systems where either the ampullae must move at constant volume (single ampullae) or they are connected to vessel systems in which the pressures are unknown but unlikely to be constant.

Quick and easy

The data graphed in this report was analyzed using a computer and custom software. Some conclusions do require quantitative analysis, which can require technical proficiency, discipline and perseverance. However, many qualitative conclusions can be made intuitively in minutes only using video viewers that are included with both Microsoft and Apple operating systems for personal computers. The major difficulty in observing ampullae behavior is that it is so slow; it's as bad as trying to see a plant grow.

This can be overcome by using the slider in a video viewer. Typically the viewer is allowed to run at the native speed, 30 frames per second, and the slider moves across the underside of the video window to indicate the fraction that has been observed. However, if you grab the slider with the mouse and pull or push it along its track, the video plays at the corresponding speed. It's generating a custom time lapse movie on the fly. Once this author discovered this technique it was always used on video files before a more methodical analysis. As any scientist knows, it is much easier to design a protocol if you know the answer.

One wonders how much movement in biological systems has not been noticed just because of a speed mismatch between observer and observed.

Conclusions

Measurement of the profiles of more than half of the 27 ampullae around the periphery of a young tunicate colony for 2000 seconds documents rhymical contractions with periods of about 80 seconds. Contractions of some colonies are quite regular in shape and timing while those of others are less so. However, in no instance is more than 50 percent of the energy in the Fourier power spectrum in the main peak. Contractions in neighboring ampullae on the periphery tend to be more correlated than those on the opposite side. Contractions in each ampullae tend to have regular timing for a time segment, then become very irregular, then snap back to regular timing. However, pairs and single ampullae surgically detached from the colony typically beat with extremely regular, constant periods and wave shapes, typically having 99 percent of the power in the main peak of the Fourier spectrum. This fact is consistent with the ampullae in a colony contracting independently with approximately equal internal timing, but mostly entrained in phase by the connecting net of blood vessels. Entrainment provides a greater reciprocal exchange of blood between the ampullae and the zooid body than would occur with randomly timed contractions. The cells in the ampullae and associated vessel net depend on nutrients from the digestive system of the zooid, and nutrients must be transported by ampullae contractions. The peristaltic heart in the zooid body reverses pumping direction at times mostly correlated with ampullae contractions. This may also increase ampullae-body blood transfer.

Abbreviations

amp: ampullae

ATP: adenosine tri-phosphate

SW: sea water

T-type: transient opening calcium channels

Declarations

Availability of data and materials

Java code for the ImageJ plugin used to measure areas and the resulting data seen in Fig. 7 etc. are available at Github.com in the repository: mwkonrad/BMC_Zoology_1.

Supplementary information

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Consent for publication

Not applicable.

Competing interests

The author declares there are no competing interests.

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Figures

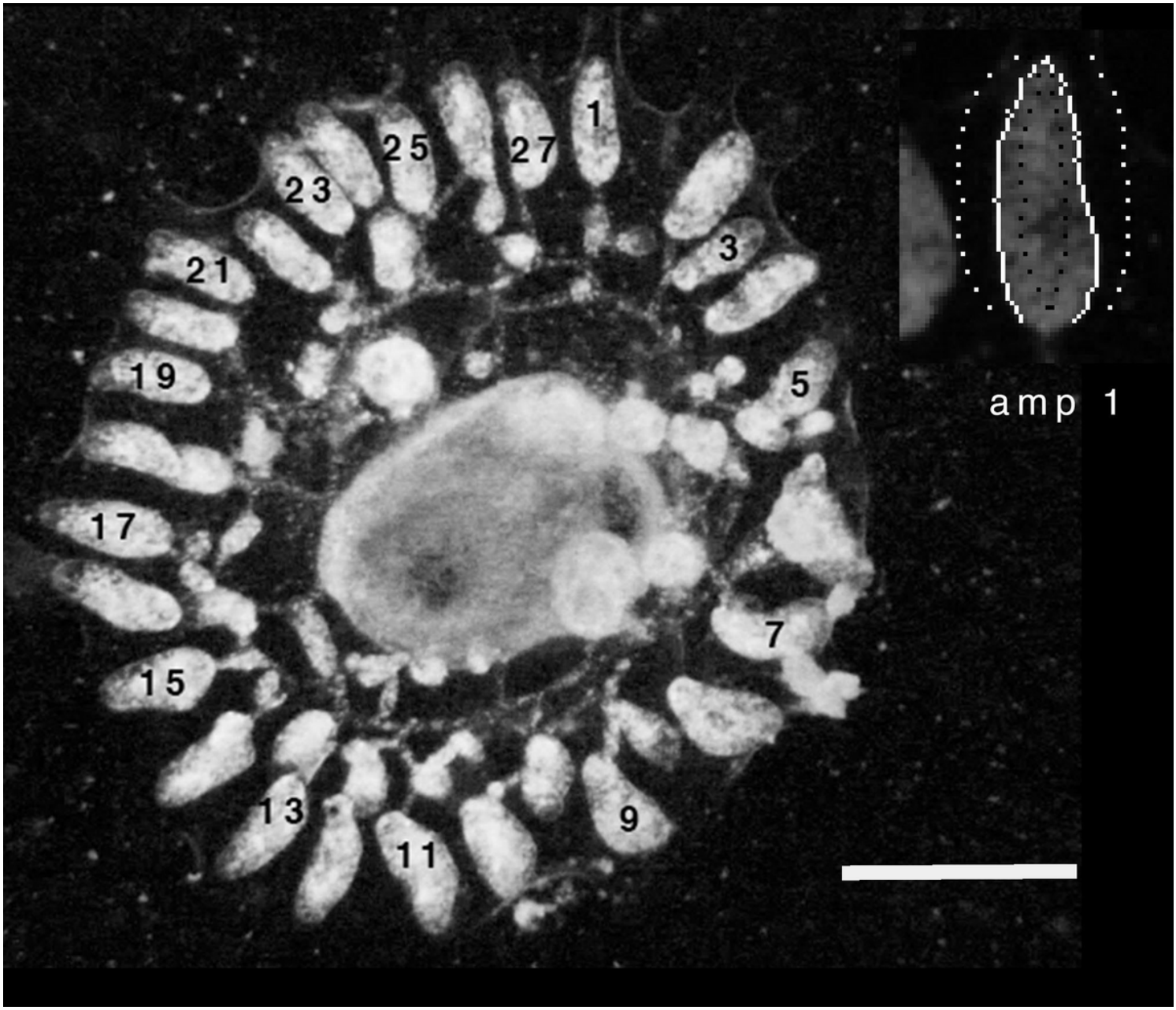

Figure 1

A typical young colony. Colonies were observed by dark field illumination and the image recorded by a video camera at 30 frames per second at a resolution of 640 by 480 pixels. This image is one frame from such a video with a field of view (FOV) of 5.6 x 4.2 mm; thus the diameter of the colony is about 3.5 mm. An identification number was assigned to each ampullae in a clockwise direction around the colony. Areas of ampullae were determined as the sum of pixels in chords across the ampullae averaged over the 30 frames in each second of the video. Scanning of each ampullae was defined by a user generated mask and the results of scans of each ampullae were curated. The scan lines for ampullae number 1 were horizontal and are indicated in the insert enlargement where the size of individual pixels can be

seen. The start and end of each scan chord are indicated by the rows of dashed white and black pixels while edges found between these limits during the scan are indicated by the two continuous rows of white pixels. The thin white horizontal lines define upper and lower quarters of the ampullae. Scale bar at lower right = 1 mm.

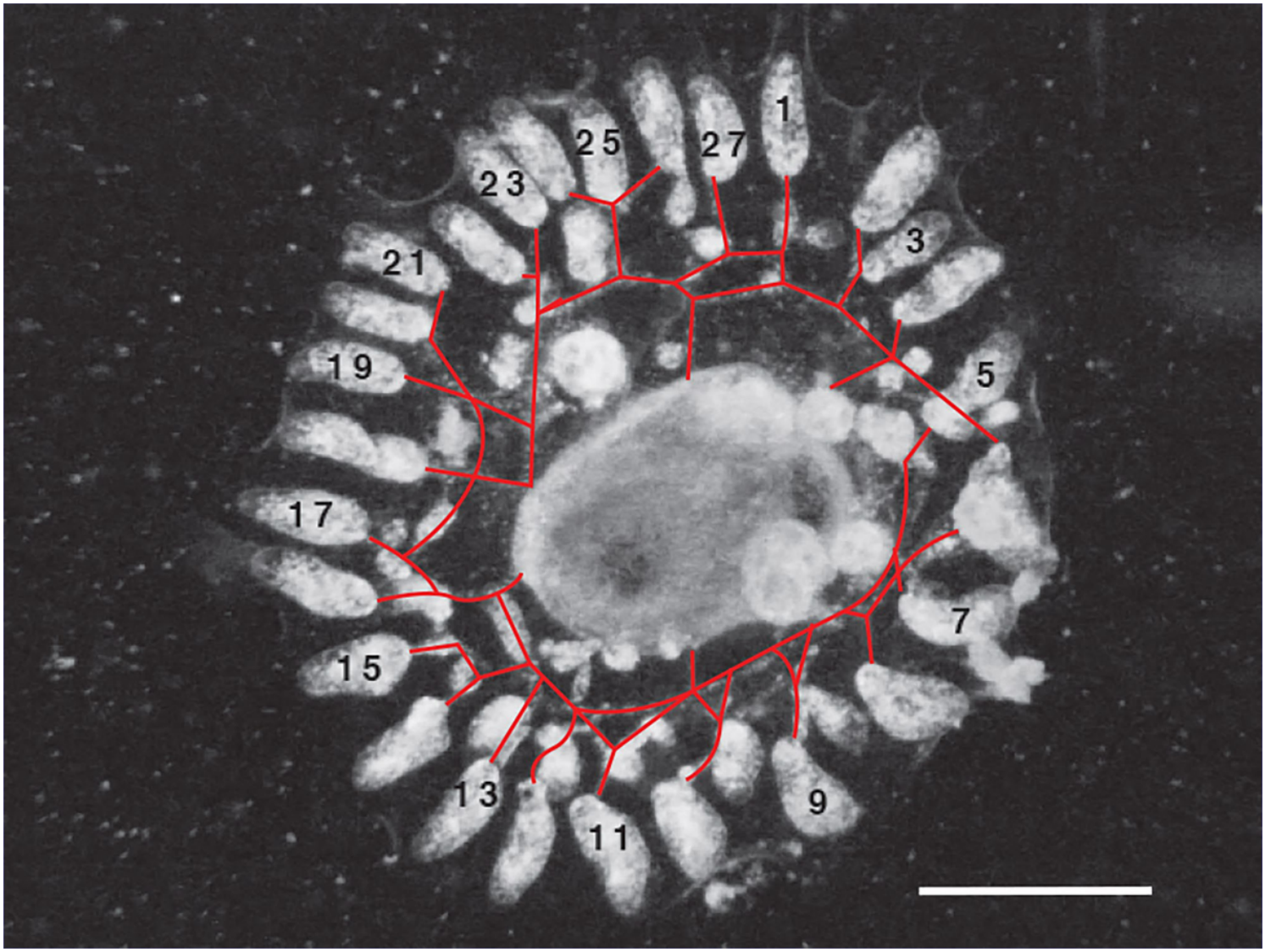

Figure 2

The functional vessel network connecting ampullae and the zooid (in red). Vessels were identified by examination of high contrast images and blood cell movement in time-lapse sequences.

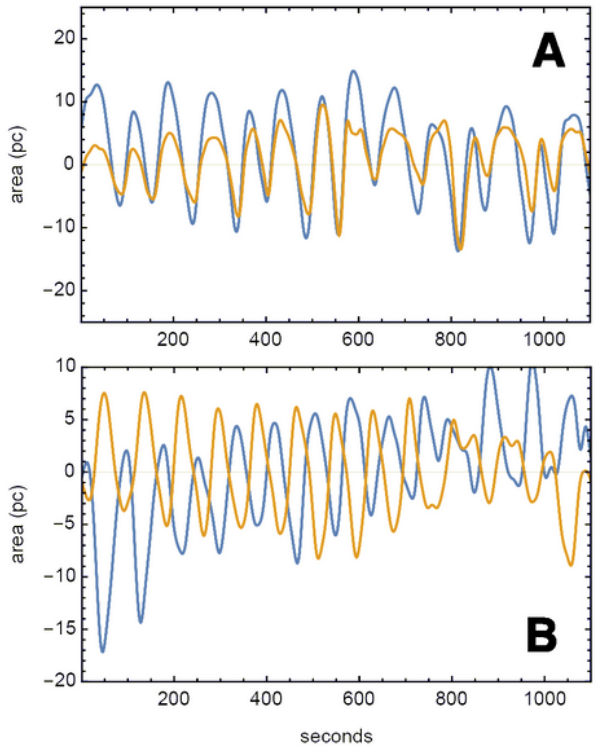

Figure 3

Contractions in segments of two ampullae. Panel A, ampullae 1 in the colony of Figure 1, ___ 2nd segment from tip, ___ 3rd segment. Panel B, an ampullae in a different colony, ___1st segment from tip, ___3rd.

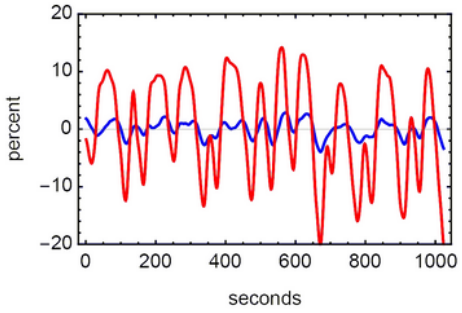

Figure 4

Width and length of ampullae 1 as percent of their means: ___ width, ___ length. The mean length was about 230 px or 2.0 mm and the mean width was about 75 px or 0.66 mm. The fractional change in width, or radius, of the ellipse is much greater than the change in length.

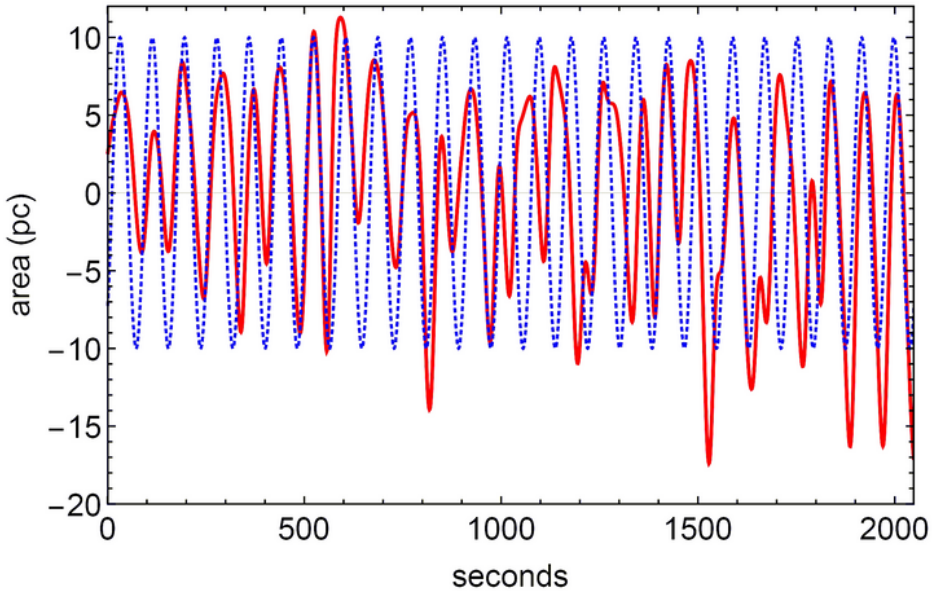

Figure 5

The contraction curve of ampullae 1 and a sine wave; ––– amp, ….. sine wave. Often the two curves are superimposable but occasionally ampullae peaks exhibit very different shapes and peak at different times.

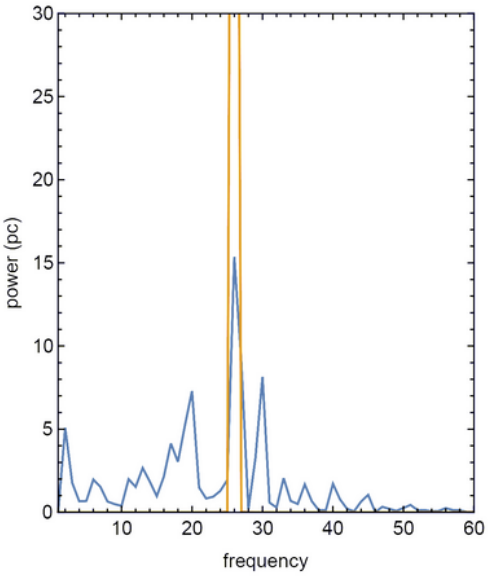

Figure 6

The Fourier power spectrum of ampullae 6 contractions; ___ ampullae, ___ sine wave

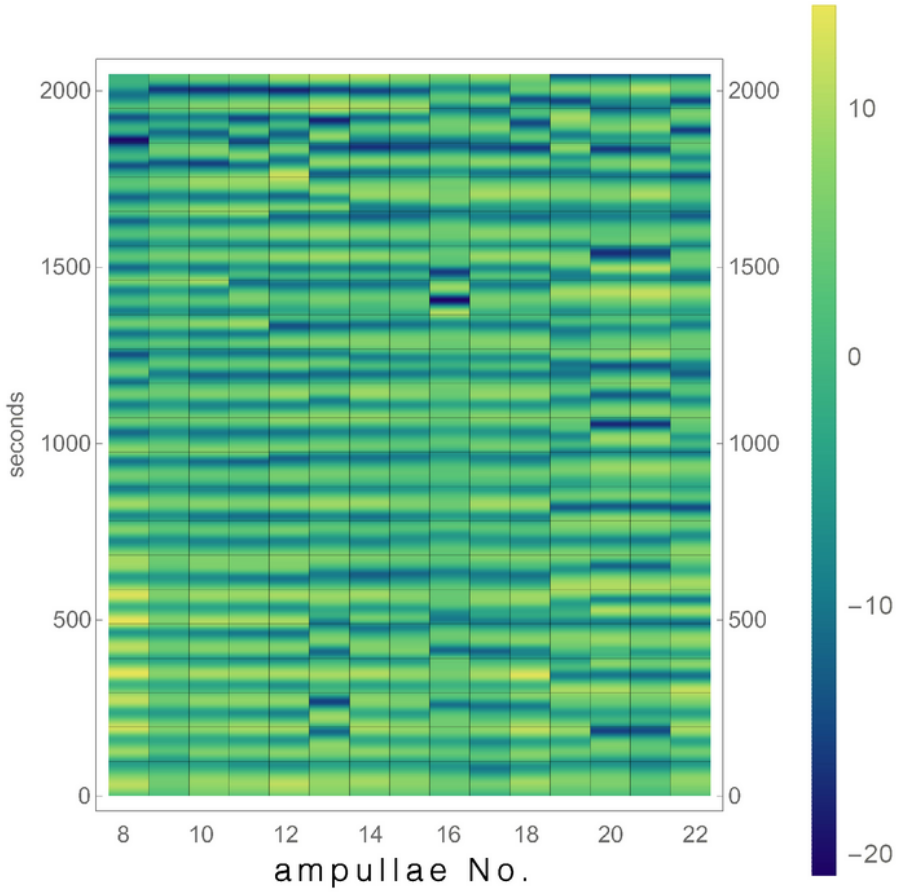

Figure 7

The percent difference in areas of 15 ampullae from their means over 2048 seconds are indicated by color, with yellow being greater and blue being smaller. The indexes of the 15 ampullae are indicated along the bottom while time for each vertical lane increases from 0 to 2048 seconds moving up the lanes. The vertical legend bar on the right gives the relation between color and percent deviation from the mean value for each ampullae.

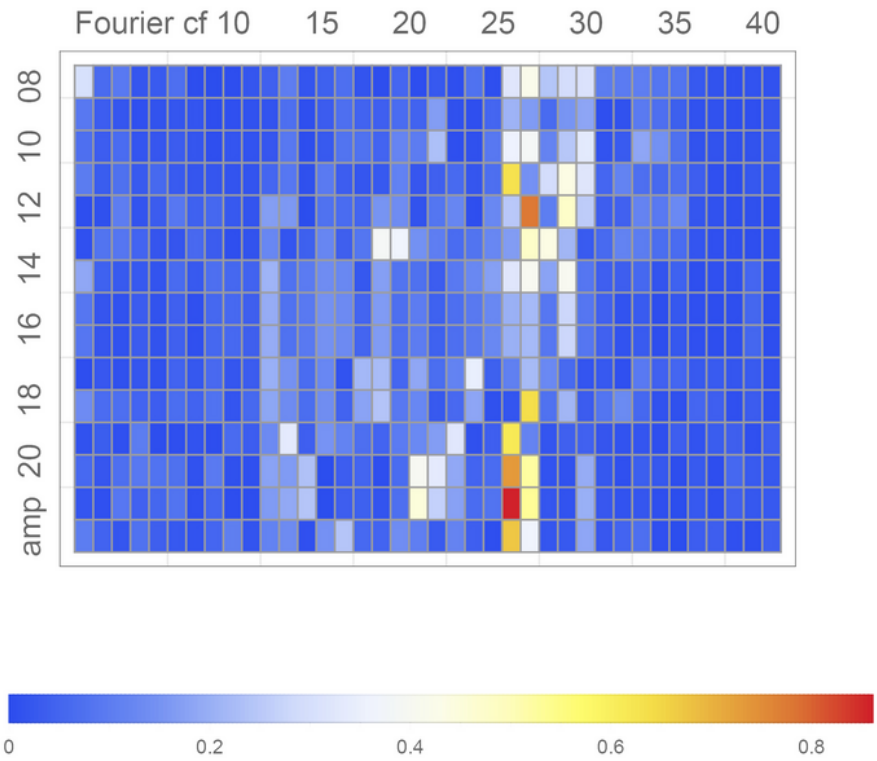

Figure 8

Terms of the Fourier power spectra for 15 ampullae are indicated by color. Lanes have not been normalized for totals of each ampullae, thus ampullae with more extreme contractions have more red cells. The legend bar on the left gives the relation between color and relative percent of total power.

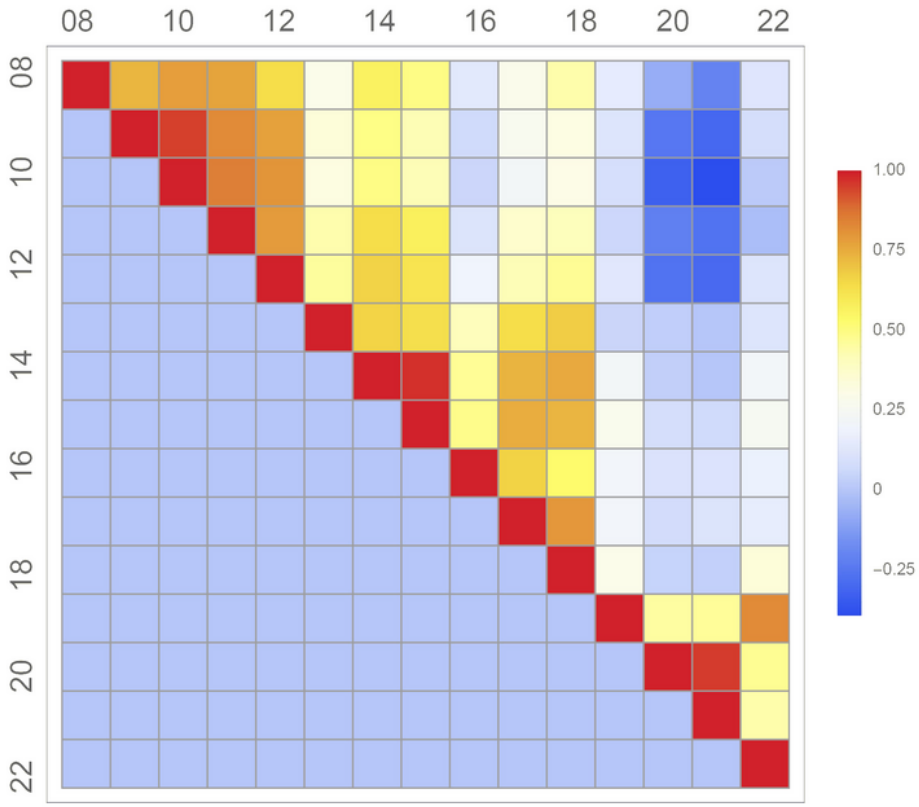

Figure 9

Correlation of contractions between all possible pairs of 15 ampullae. The bar legend on the right gives the relation between correlation and color.

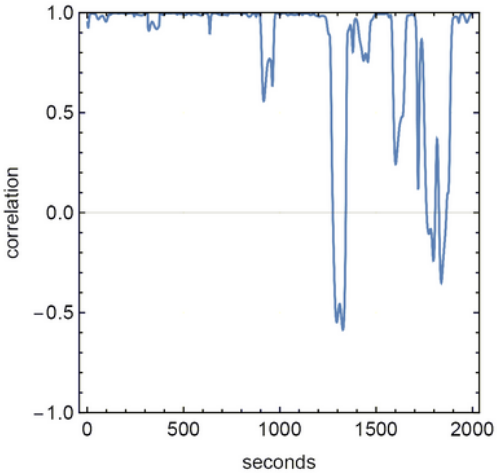

Figure 10

Contractions of ampullae 11 and 12 are seen in the top panel and the rolling time correlations between the two over successive 60 second periods are seen in the lower panel.

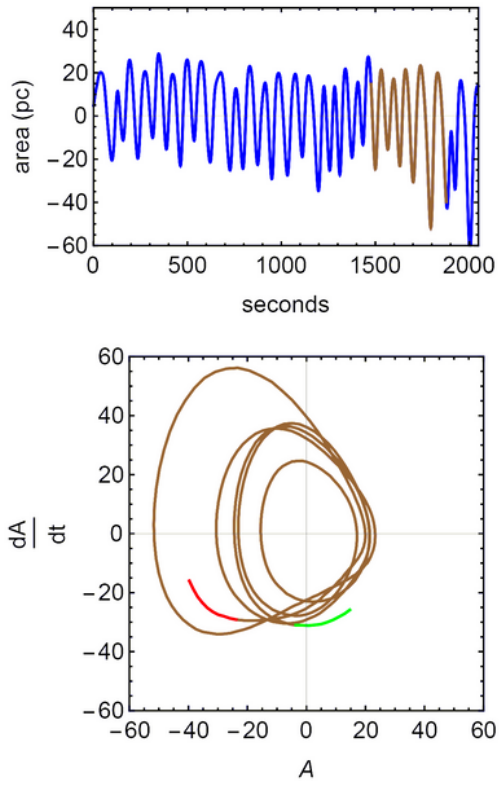

Figure 11

The phase projection of ampullae 9 over 400 seconds. The entire 2048 second profile is seen in the top panel, with the 400 second segment in brown. The phase projection in the lower panel, with the start in green and the end in red. The area and its derivative have been normalized to percent of maximum range.

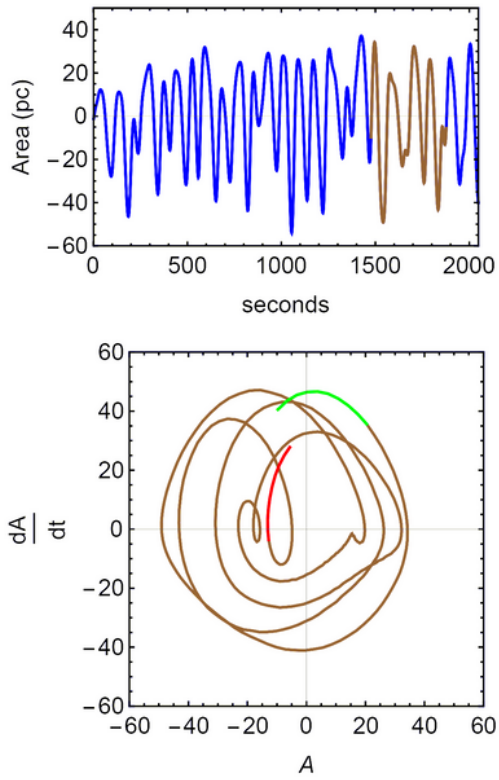

Figure 12

The phase portrait of ampullae 21 over the same 400 second period described in Fig 11. Colors have the same meaning as in Fig. 11.

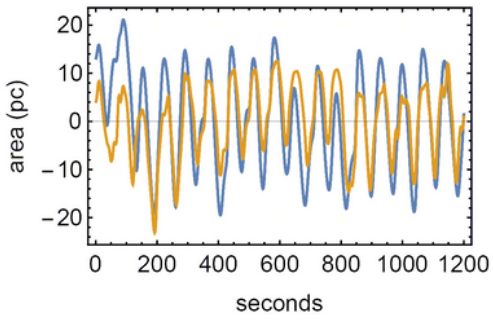

Figure 13

Contractions of a vessel and its attached ampullae; ___vessel, ___ ampullae. The vessel segment scanned was approximately 1/3rd the length of the ampullae and started this distance from the ampullae-vessel junction.

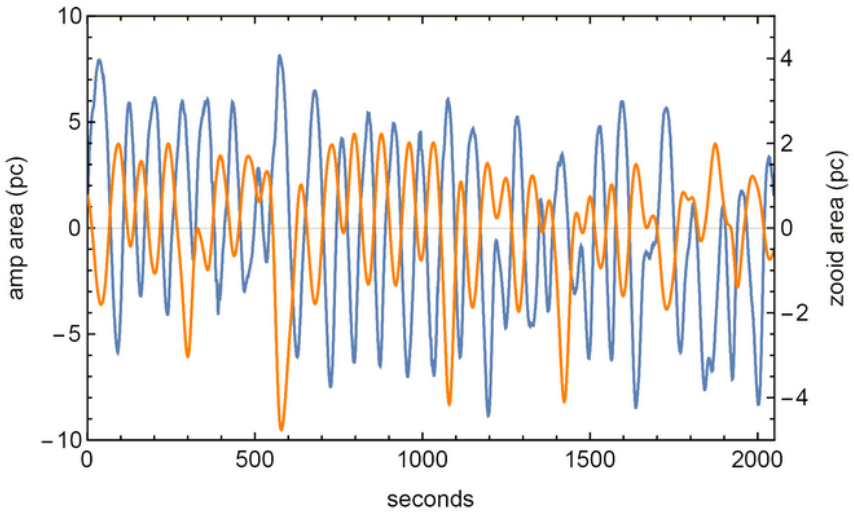

Figure 14

Contractions of zooid compared to ampullae. ___ zooid, ___ amp The area of the zooid and the average of 19 out of the 27 ampullae of the colony seen in Fig 1.

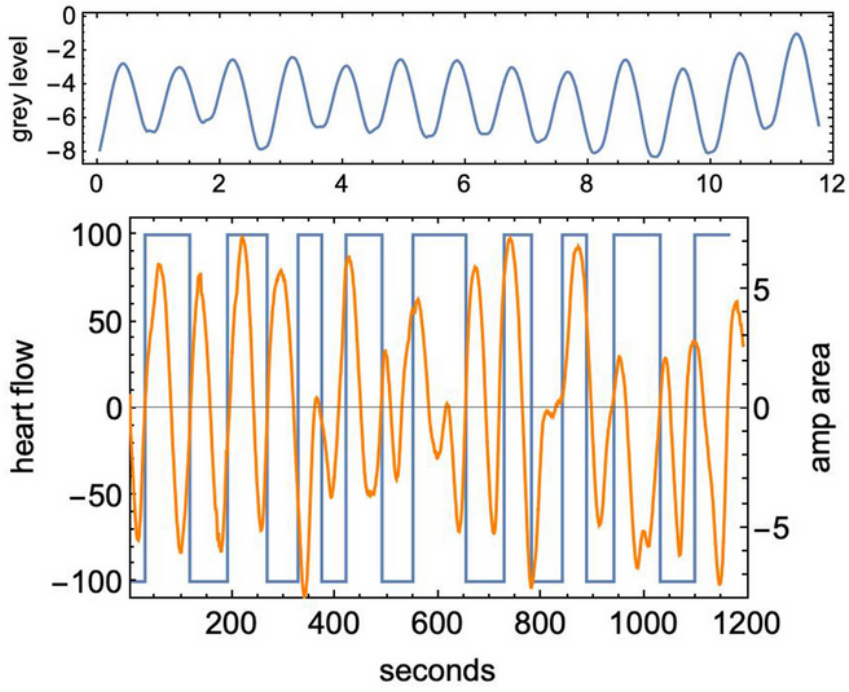

Figure 15

Entrainment between ampullae contractions and reversals in the peristaltic heart. The top panel displays 14 beats ____ of the peristaltic heart during a 12 second period, giving a period of 0.8 seconds. The beats were detected as variations in grey level in a video with no resolution of the structure of the heart. The bottom panel shows contractions of 12 ampullae ____ and blood flow generated by the heart ____ during

1200 seconds, 100 times the length of the top panel. During most of the 1200 seconds reversals of the heart occur at or near the maximum ampullae area, consistent with a 1:2 entrainment.

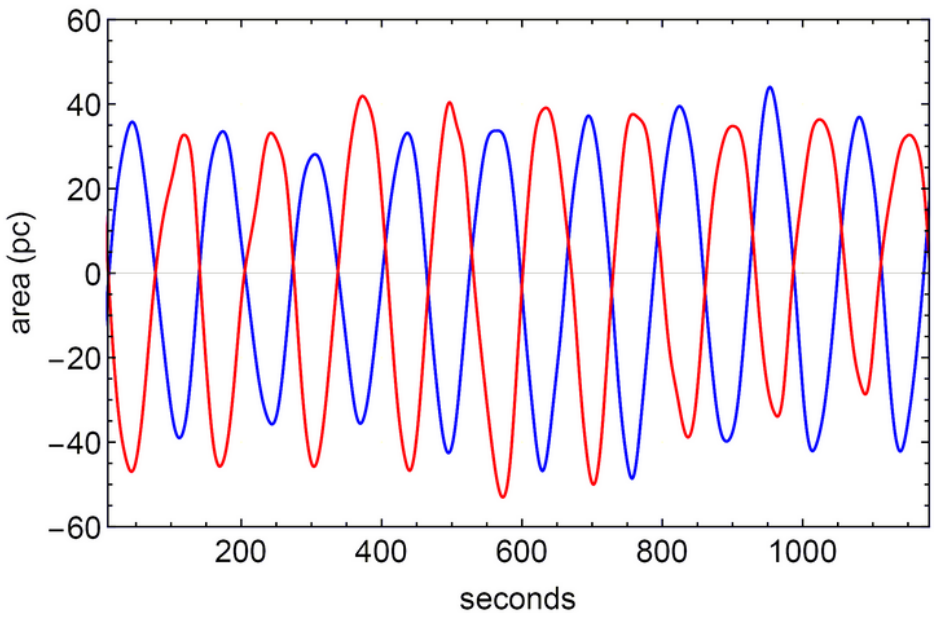

Figure 16

Contractions of a pair of detached ampullae. ___amp 1, ___amp 2. A pair of connected ampullae were surgically separated from a colony. The area versus time curves, trimmed to the cross over times and

normalized to percent of range for each, are shown in the top panel. The phase portrait of ampullae 1 is displayed in the bottom panel. The green segment is the beginning and the red is the end of the trajectory.

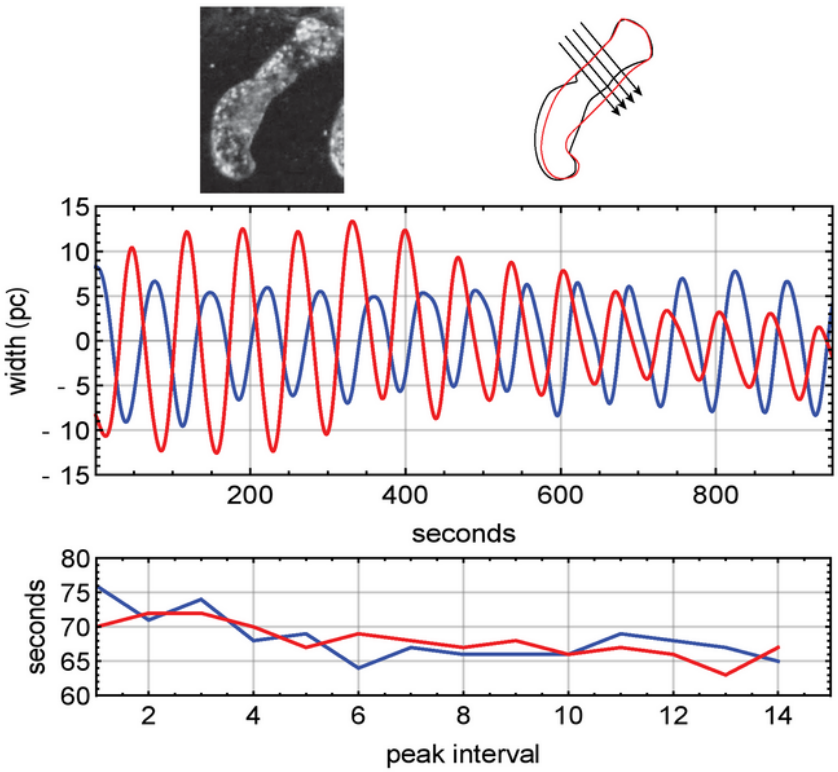

Figure 17

Contractions of single detached ampullae. The image of one detached ampullae is seen in the small upper left panel. The severed vessel that had connected the ampullae to the colony circulation is at the upper right end. A diagram in the small panel to the right displays outlines in blue ____ and red ____ of two

most extreme shapes. Oscillations were observed by scanning a quarter of the ampullae, as indicated by the four arrows. The middle panel displays the results of such scans of ampullae 1 ____ and 5 ___ in a consecutive series of 6 along the circumference of a colony. The time in seconds between each peak is plotted in the lower panel.