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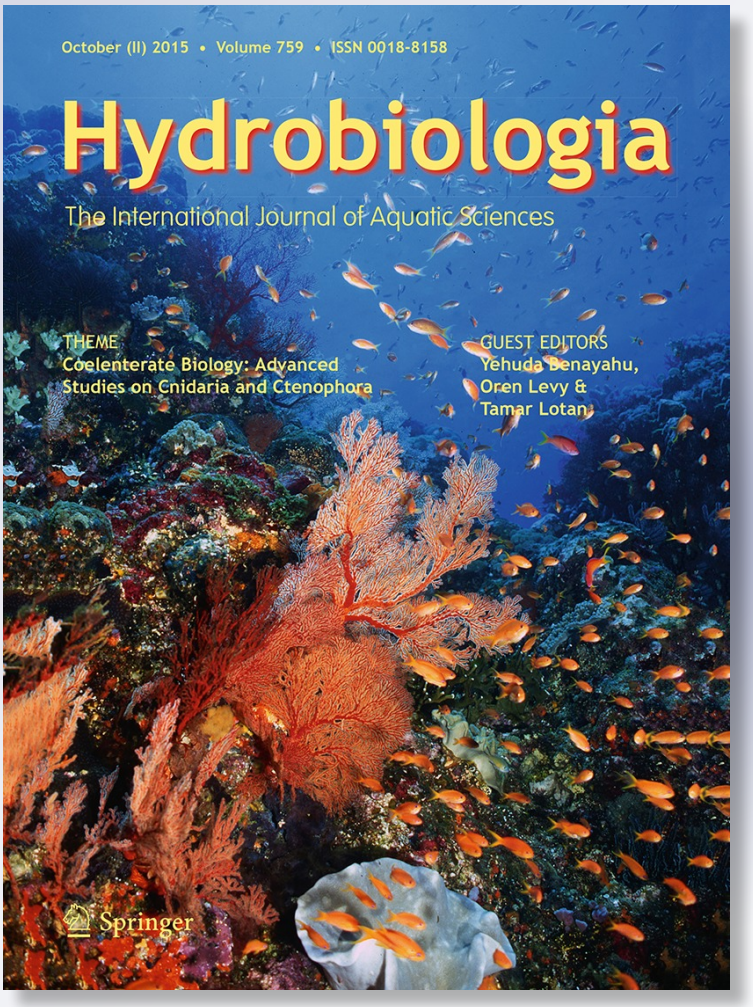

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COELENTERATE BIOLOGY

A new method for studying the transport system in colonial hydroids

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Abstract Hydroplasmic flow (HF) is clearly visible in most colonial hydroids with transparent or translucent body walls. It is difficult to study the transport system in colonial hydroids without simultaneous recording of HF in different parts of the highly branched colony. We have developed a method for integrated characterization of the transport system using video data collected in a single location in the transport system. Video recording is conducted at the frame rate of 4–30 FPS. When processing the results, both the lumen diameter in two cross sections of the coenosarc within the same frame and the rate of particle flow in the hydroplasm are taken into account. These data help to factor in the dynamic aspects of the following parameters: the rate and direction of HF; the presence/absence of peristaltic waves of contraction/expansion of the coenosarc; the volume of transported hydroplasm; the distance of particle transfer in a single cycle of HF. The combination of these parameters makes for sufficiently detailed characterization of the transport system in general, and its reaction to experimental impact. We have used this method to explore the frequency of coenosarc pulsations, the rhythm and range of HF, and the level of colony integration.

Keywords Hydrozoa · Hydroplasmic flow · Colony integration · Gastrovascular cavity · Periodic pulsations

Introduction

The transport system in hydroids presents a unique example of a system with decentralized self-organization of multiple pulsators contributing to the propulsion of hydroplasm along the extensive and highly branched closed-loop circuit of the tubular coenosarc. Such objects can be used to study the processes of self-organization, the system's reactions to changes in its architectonics, and the degree and efficiency of integration in a modular organism depending on the system's various morphological parameters and its functional state. Some species of colonial hydroids have thin transparent walls and can be cultivated in the lab; this implies opportunities for observing the processes of growth, morphogenesis, cell migration, and food transport and digestion in intact specimens, which cannot be achieved with most other objects unless bulky equipment is employed. However, one methodological problem has remained a challenge until now: namely, the study of multiple

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interconnected acting parts of the same body, especially when the organism is highly branched and its parts are functioning independently of each other, for the most part, e.g., hydranths in a hydroid colony. A transport system that encompasses the whole colonial organism is a challenging object for researchers, due to an absence of techniques for synchronized recording of the activity of multiple pulsators that contribute to the transport of hydroplasmic flows (HFs) as such in different parts of the colony.

The purpose of this paper is to present a newly developed and tested method for evaluating the efficiency of transport systems in hydroids on the basis of several indicators, and using records of pulsation activity of the distal parts of the coenosarc described by different authors (Berrill, 1949; Hale, 1960; Fulton, 1963), as well as the rate and direction of hydroplasmic flow in one location in the colony, as opposed to monitoring several locations at once. This method differs from previously described versions (Marfenin, 1985a; Blackstone, 1996) in that it enables the evaluation of interaction between hydroplasmic flows and coenosarc pulsations in two closely spaced cross sections.

Most hydroids form colonies, i.e., hydranths interconnected by a tubular body, also known as coenosarc, which is distinctly visible and shapes the structural framework of the transport system. Stolons and upright stems are parts of the shared colonial coenosarc. The hydroid colony is a product of individual development, a modular organism with numerous homotypic parts (modules), which rarely contains unique non-recurring organs and lacks any physiological centralization. There is no single designated propulsion organ (like the heart), and none of the parts of the colonial body perform dedicated functions of coordination and control. The nervous system is extremely primitive. Integration is achieved through parity-based interaction of equivalent parts and self-organization. A colony is formed as a result of modular growth, i.e., cyclic (recurring) morphogenesis (Marfenin, 1999, 2008) and branching. Therefore, the transport system should be viewed as an ontogenetic whole, it develops gradually, it reaches a significant extent and level of branching, and the gastric cavity of the coenosarc is shared by the whole colony and connected with the gastric cavities of all hydranths. This cavity of the coenosarc is filled with fluid—the hydroplasm, which can travel great distances commensurate with the size of the colony. It ensures the delivery of food, fluid, and metabolites from one part of the colonial organism to another, mainly from areas of food ingestion to growth regions (Karlsen & Marfenin, 1984; Marfenin, 1985a; Burykin, 2008, 2010). This, in turn, serves to express the physiological integration of a colony, which can be successfully used to quantify the efficiency of the transport system, i.e., the duration of food transport and distance traveled from areas of food capture and initial digestion to areas of final digestion, as well as growth and active branching.

Earlier experiments with local feeding of the proximal stems of *Dynamena pumila* and *Gonothyrea loveni* have demonstrated that limited food intake restricts growth to distal stems and the stolon itself (Marfenin & Burykin, 1979; Marfenin, 1993). The number of growing stems strictly correlated with the amount of food absorbed daily by proximal stems. Berrill (1949) was the first to show this using a more basic experiment.

In another study, we demonstrated that in the process of colony growth in *D. pumila* a constant ratio is maintained between the number of hydranths, the length of hydrorhiza and the number of growing tips (Marfenin, 1995), which implicitly confirms the whole-colony self-regulation hypothesis. Both findings inspired further research into the physiological basis of colony integration.

The functioning of the transport system in hydroids has been discovered to be of two types: either (a) continuous unidirectional transport of hydroplasm, or (b) movement of hydroplasm in alternating opposite directions.

Continuous unidirectional movement of hydroplasm is caused by the activity of gastrodermal flagella and can only be found in those hydroids where the longitudinal mesentery divides the gastrovascular cavity of the coenosarc into two or more canals (*Tubularia*, *Corymorpha*). The hydroplasm flows continuously and at a constant rate in one direction along one of the canals, and continues its movement with the same velocity in the opposite direction. The two canals connect to each other near hydranths and growing tips, i.e., in the terminal segments of the tubular coenosarc where the longitudinal mesentery ends before it reaches the end of the tube (Marfenin, 1985b). The functioning of this type of transport system is rather straightforward and leaves no room for ambiguity.

The other type of transport system is more complex and not as easy to understand. The hydroplasm flows along the only canal of the coenosarc with varying speed, and in alternating directions. Apart from direction and rate of flow, these hydroplasmic movements also vary by such parameters as duration of unidirectional flow, and volume of transported hydroplasm. The latter depends not only on the rate and duration of flow, but also on the variable diameter of the coenosarc lumen. Hence the use of different attributes—"long-distance" versus "short-range", "rhythmic high-speed" versus "non-rhythmic low-speed", and "powerful"—to describe hydroplasmic flows. The choice of attribute depends on which parameter needs to be emphasized at each particular stage of description or discussion of results, e.g., rate of flow, distance, rhythmicity of HFs, or volume of transported hydroplasm.

Due to the presence of food particles in the hydroplasm one can observe their movement under a microscope in either one or the opposite direction, at accelerating or declining rate or in «surges». A change in the lumen of the coenosarc is easily distinguished, as it becomes dilated with the approach of the HF and occluded toward the end of the passage of hydroplasm. Gastrodermal flagella create rotational flows of hydroplasm, but are unable to propel it over significant distances (Berrill, 1949; Karlsen & Marfenin, 1976, 1984; Burykin, 2010). Radial contractions of the coenosarc and the hydranths seem to be the primary engines of hydroplasmic movement, although the pattern of interaction between pulsators is poorly understood (Marfenin, 1985a). Multiple uncoordinated pulsations generate independent flows of hydroplasm. However, occasionally one can observe rapid long-distance HFs instead of short-range transfer of particles in the hydroplasm. These long-distance flows are likely caused by the interaction of scattered individual pulsators. This type of transport system has been termed «pulsatory-peristaltic», and the hypothetical mechanism of interaction between pulsators that drives long-distance HFs, and ensures the physiological integration of a colonial organism has been described (Marfenin, 1985a, 1988). Colonial hydroids with the pulsatory-peristaltic transport system can be successfully used as an experimental model of self-organization in biological systems, provided that a simple and reliable method is available for capturing and analyzing HFs.

Material and method

The method for determining the key parameters and efficiency of HFs is based on long-term imaging technology used to capture two parameters in a single location: (1) radial pulsations of the coenosarc; and (2) direction and rate of HFs.

Species with colorless coenosarc are preferable for the purposes of transport system research. Colonies should be cultivated on glass slides using the method described by Fulton (1960). Newly hatched *Artemia* nauplii are fed to the colony either 2–3 times a week in a separate vessel for 1–2 h at a time, or added to the aquarium daily, but in smaller amounts (about 1–3 per ml). Overfeeding leads to rapid growth of the colony, but undermines the visibility of HFs as the coenosarc becomes less transparent. Underfeeding, on the other hand, leads to slower growth and thinning of the coenosarc, but improves the in vivo visibility of coenosarc cells and the movement of hydroplasm. Suspended particles are always present in the hydroplasm. Even if a colony has been deprived of food for a long time, its hydroplasm will contain particles—its own cells that enter the gastric cavity as a result of tissue regression—a process or dedifferentiation of hydranths and terminal segments of the coenosarc (Braverman, 1969, 1973; Hale, 1960).

Transparency of the coenosarc declines proximally (from the growing tip of the stolon or stem toward its base). It is therefore easier to record HFs in the newer segments of stolons and stems. Continuous recording of images in one location for at least 1 h, but preferably 2–3 h or more, serves to capture the following processes:

- Rhythmic high-speed HFs usually with a period of 15–20 min or more, against the background of non-rhythmic low-speed HFs;
- Radial pulsations of the coenosarc (or hydranth), not in one location, but in several locations within the same frame, which helps to determine the direction of the wave of coenosarc contraction/expansion, if any;
- The front of HF, i.e., the moving boundary between the regions of hydroplasmic quiescence and flow; the front of HF usually spreads ahead of the hydroplasmic current;
- The movement of individual smaller and larger food particles and particle accumulations in the hydroplasm, within the same frame; sometimes the particles move in opposite directions;

- The sticking of larger particles to the surface of the gastroderm; the tearing off of said particles by strong hydroplasmic flows.

Time-lapse videos can be recorded either with special cameras intended for scientific application, or with video surveillance cameras, e.g., AV100 by Arecont Vision. Recommended frame rate is 4 FPS, but in some cases, when dealing with high-speed HFs, the frame frequency needs to be increased. It is also recommended to take a panoramic image of the whole object by dragging it under the camera lens. The microscale needs to be photographed before and after the video session, for calibration of the screen scale that will be used for all measurements.

Apart from the use of standard procedures for object description and control of key environmental variables (seawater temperature and composition, salinity, frequency of water change or water turnover time in the flow-through mode, pre-experiment cultivation conditions, duration of experiment), experimental study of colonial hydroids also relies on indicators specific to modular organisms, such as:

- Schematic diagram of the colony structure showing: (1) the stems, (2) distances between them, (3) the length of the distal segment of the stolon from the newest stem, (4) locations of lateral stolon outgrowth, (5) number of internodes on each stem, and (6) state of the growing tips of the stems;
- Size of the colony (number of stems, number of internodes in the stems, length of stolons);
- Share of resorbed hydranths (if they are visibly distinguished, like in *Campanulariidae*);
- Location and number of growing tips;
- Feeding periodicity with indication of the last feeding session prior to the beginning of the experiment, the amount of food intake;
- The location of hydranths that received food often has to be indicated as well, since in colonies (unlike solitary organisms) this factor affects the functioning of the transport system;
- Spatially differentiated growth increments (for all stems and stolons) per unit of time before and during the experiment serve as important indicators of the condition of the colony.

The feeding regime and water temperature need to be specified at least a day before video recording begins, in consistence with experiment objectives.

Obtained video recordings can be processed with the help of image recognition software (Blackstone, 1996), however, we preferred visual processing of videos, with parallel collection of additional information. The following need to be determined: (1) sampling rate for frame processing, e.g., every 100th frame; (2) fixed locations within the frame where lumen values are obtained; (3) order of magnitude and margin of error adopted when measuring the path of particles in the hydroplasm.

We recommend that four parameters are measured: diameter of the coenosarc lumen in two cross sections spaced apart within the same frame, the rate and direction of hydroplasmic flows. The rate of HF is arbitrarily equated to the pathline of particles per field of vision in 1 s (Fig. 1). When frame frequency is set to 4 FPS, the shifting of particles per field of vision through 4 frames is measured. It is easier to register the path of larger particles commensurate with the lumen of the coenosarc (usually 2–4 times smaller), or easily discernible particle accumulations.

Measuring only four parameters helps to obtain an informative range of indicators and indices, namely:

1. Distance traveled by HFs over one cycle of unidirectional movement of hydroplasm, which characterizes the scale of physiological integration of the colony;
2. Periodicity of HFs as an indicator of dependence on one dominant pulsator, or as an indicator of synchronization of multiple pulsators; period of HF is calculated as the period between maximum peaks in each one-way phase (toward the tip or in the opposite direction);
3. Number of reversals in the direction of HFs within one cycle (between two consecutive strong and rapid unidirectional currents), as an indicator of the number and impact of individual pulsators that affect the HF;
4. Comparison of distal and proximal hydroplasmic flows in terms of amplitude, duration, and periodicity;
5. Periodicity of coenosarc pulsations;
6. Correlation between coenosarc pulsations in two monitored cross sections within the same frame, which help to determine the degree of independence of their pulsations, as well as peristaltic waves of coenosarc contraction and expansion, if any;

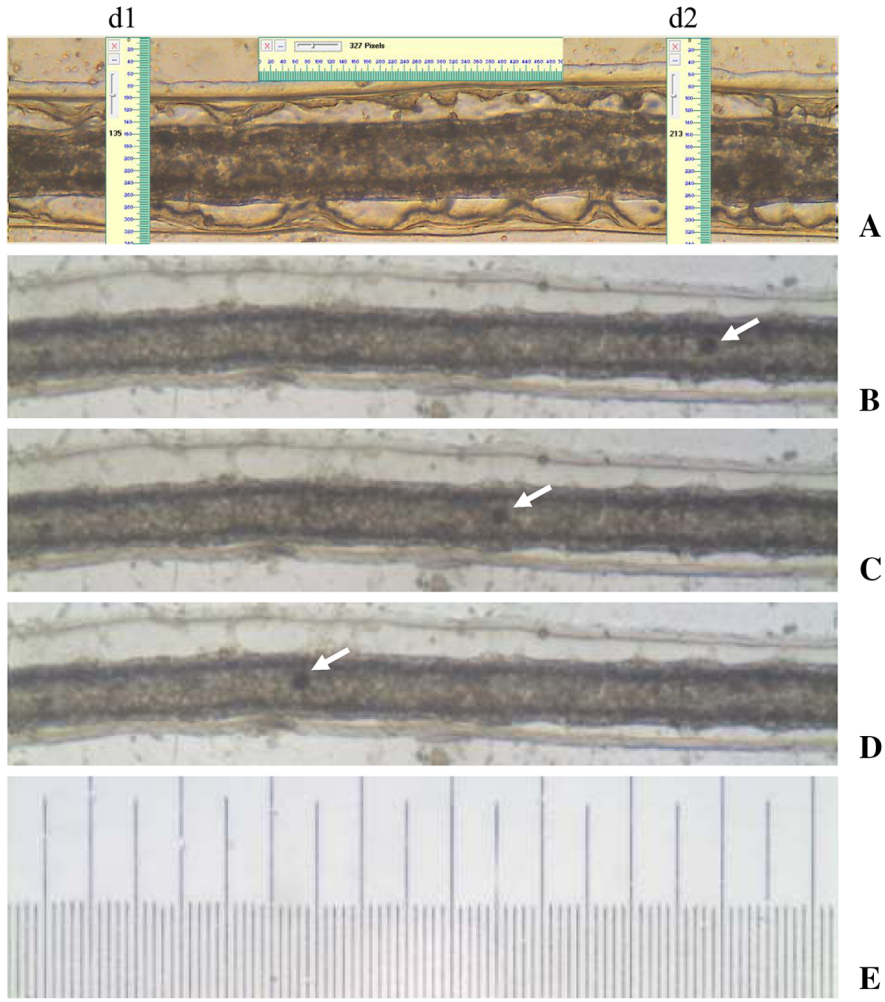

Fig. 1 An example of registration of radial pulsations of *G. loveni* coenosarc at d1 and d2 (**A**); hydroplasmic flow dynamics (**B**–**D**), scale: 0.9 mm (**E**); *arrows* a moving food particle

7. Correlation between coenosarc pulsations and hydroplasmic flows;
8. Amplitude of local coenosarc pulsations as an indicator of the potential role of a particular segment in the propulsion of hydroplasm;
9. The impact of environmental factors and experimental treatment (including changes in the colony configuration) on the functioning of the transport system;
10. Efficiency of the transport system depending on the feeding regime, etc.

To illustrate the use of the proposed method, we present the results of time-lapse video recording of coenosarc pulsations and hydroplasmic transport near the growing tip of a lateral "arm" (an arm is one outgrowing stolon with all associated stems) of a large colony of *G. loveni* before and after it was severed from the rest of the colony.

Figure 2 shows the colony structure schematic, including the location of time-lapse video acquisition. The colony consists of a total of 92 stems, which contain over 300 internodes (standard segments of coenosarc ending with hydrothecae). The severed arm has only 7 new stems with a total of 13 internodes. The stolon tip was growing.

The colony was grown on a 9 × 12 cm glass slide from an isolated stem; for a month it was cultivated in the laboratory in a temperature-controlled aquarium with water recirculation at 16–18°C. Newly hatched *Artemia* nauplii were fed to the colony for 1–2 h daily. Content of nauplii was maintained at 0.5–2.0 per ml. Final feeding should occur at least 12 h before the start

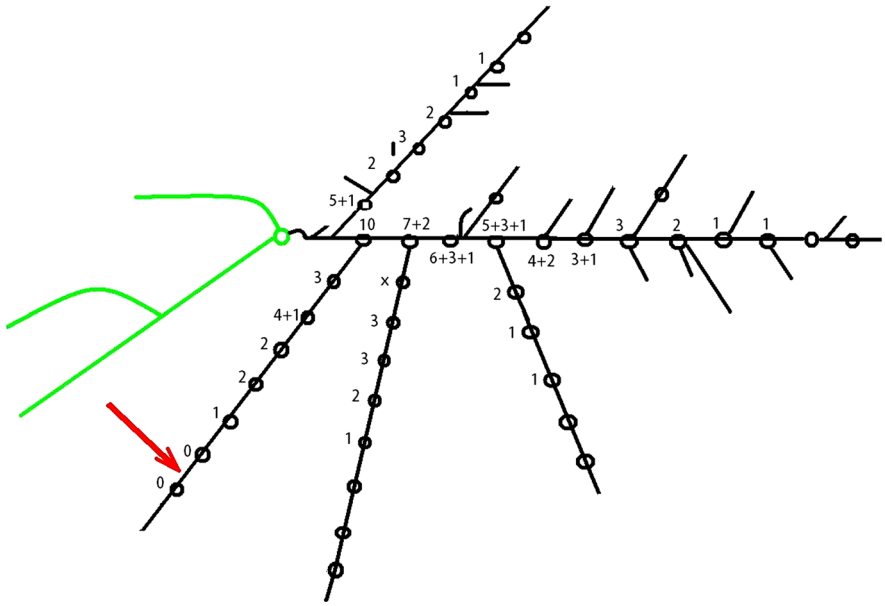

Fig. 2 Schematic of a colony of *G. loveni*: *straight lines* stolons; *circles* stems; *figures* the number of hydrant *h*s; *arrow* location of observation and video acquisition; *curved line without circles* the parent colony on the reverse side of the glass from which the arms of the observed colony radiate

of video recording, unless a specific feeding regime is prescribed by experiment objectives. In the example below, the last feeding of the colony took place 30 h before the experiment. Time-lapse videos were recorded in a cuvette at 16–17°C filled with 150 ml of fresh seawater without changing it during the whole experiment. Videos were recorded for 3 h 20 min before the stolon was cut off; recording was resumed 24 min after the stolon was cut off, and continued for 2 h 25 min. Findings are shown in Figs. 3, 4, 5 and Table 1. (To facilitate comparison, Figs. 3, 4 and 5 show 2-h recording periods).

Results

The following presents a sample interpretation of the results using proposed indicators.

When an "arm" is cut off from the colony, it results in immediate and marked stabilization of coenosarc pulsations and intensification of HF rates (Figs. 3, 4, 5) observed for at least 4 h after the surgery.

After 24 h HF pulsations display the same rhythmicity and intensity, while coenosarc pulsations become desynchronized, which is manifested in weak periodicity and lack of coordination between pulsations of vicinal segments of the coenosarc (Fig. 6).

Immediately after the surgical removal of an arm from the colony:

1. Duration of quiescent periods in the movement of hydroplasm (resting phase) declines considerably (Table 1, line 7);
2. And percentage of HF registrations towards the tip of the stolon increases, an indication of reinforced role of the distal end of the stolon as the receptacle of hydroplasm during pulsations (Table 1, line 8);
3. The average amplitude of HF increases 1.5–2.5 times both in the distal and in the proximal directions (Table 1, lines 10 and 11). Amplitude of hydroplasmic flows is the maximum rate of HF registered in the course of one half-cycle (positive, i.e., distally directed flow, or negative, i.e., proximally directed flow);
4. Maximum values of HF rate also increase more than twofold (Table 1, lines 12 and 13);
5. Immediately after the "surgery" no changes are detected in the periods of coenosarc pulsations (Table 1, lines 5 and 6) and HF pulsations (Table 1, line 15), i.e., observed changes in the functioning of the transport system are not related to shifts in the periodicity of pulsations; however both become more stable, judging by the changing values of standard deviation (SD);
6. At the beginning of the experiment the periods of HF pulsations are equal to periods of coenosarc pulsations, both before and immediately after an "arm" is cut off the colony, but 24 h later they are out of sync (Table 1, lines 5, 6 and 14, 15), which

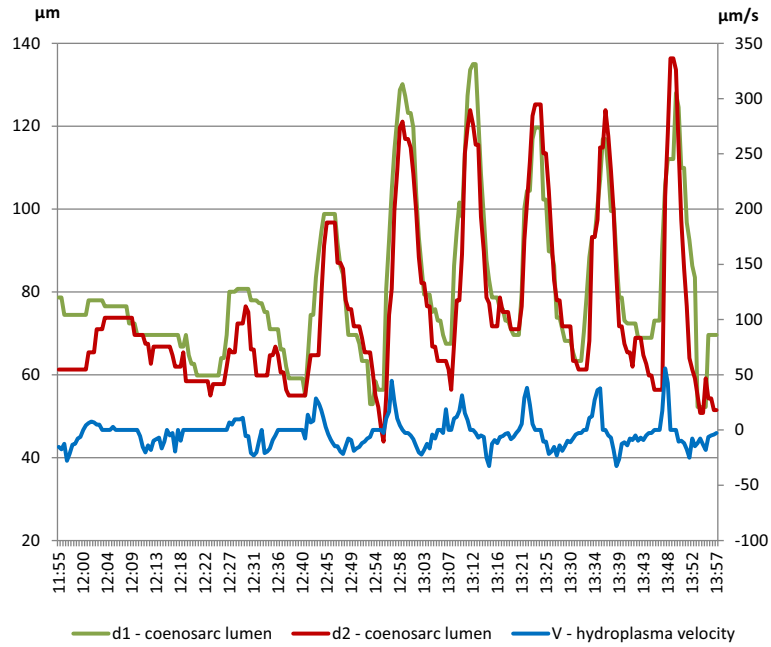

Fig. 3 Dynamics of: *1*) coenosarc pulsations in two cross sections (d1 and d2), and *2*) the rate of hydroplasmic flow (+hydroplasmic flows = towards the tip) before the arm of the colony was cut off. (Section of a stolon of *G. loveni* between *1* and *2* stems nearest to the stolon tip)

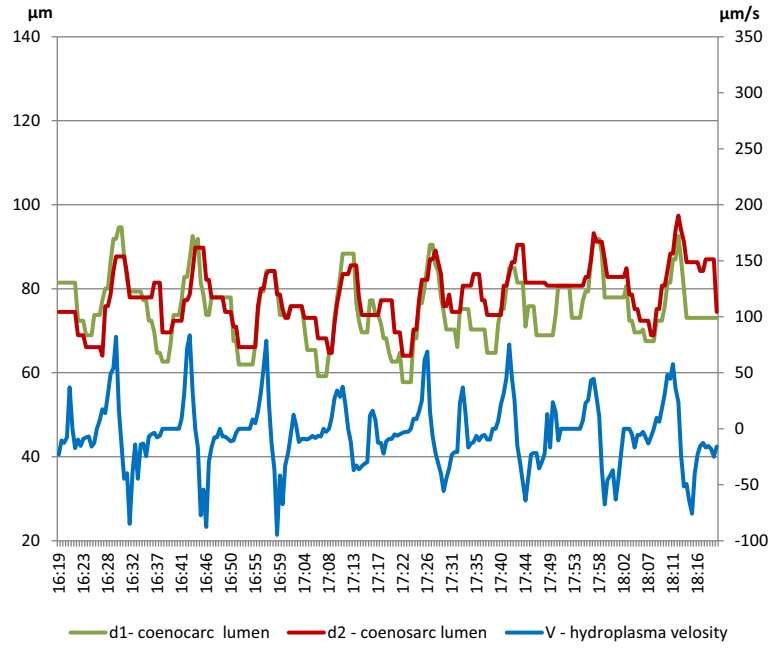

Fig. 4 Dynamics of: *1*) coenosarc pulsations in two cross sections (d1 and d2), and *2*) the rate of hydroplasmic flow immediately after the arm of the colony was cut off. (Section of a stolon of *G. loveni* between *1* and *2* stems nearest to the stolon tip)

can be interpreted as a sign of desynchronization in the performance of the transport system;

7. Meanwhile, variability of coenosarc pulsations increases after 24 h (Table 1, lines 5 and 6);
8. Which is supported by changing values of Correlation Coefficient (**CC**) for d1 and d2, corresponding to correlation between pulsations of coenosarc segments within the same stolon internode (Table 1, line 16);
9. Even though correlation between pulsations of the coenosarc and HF pulsations remains marginal (Table 1, lines 17 and 18).

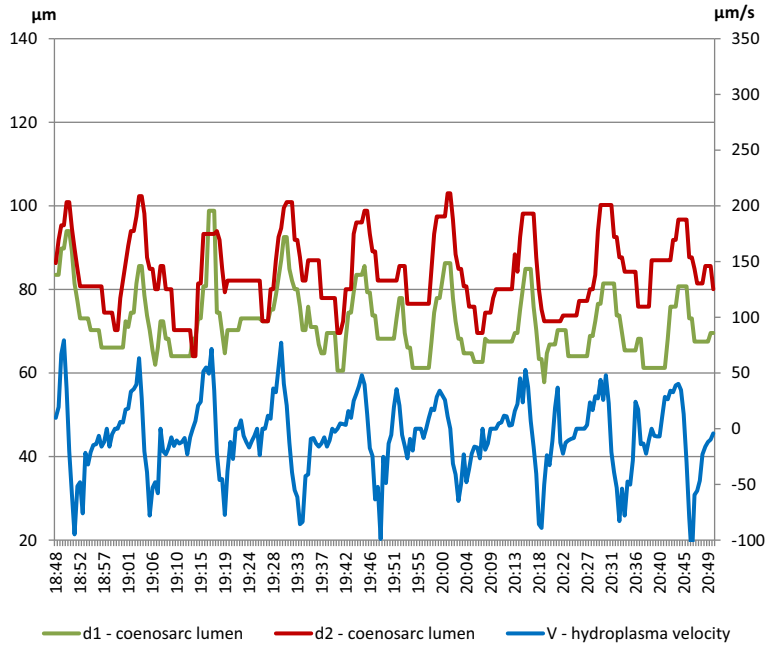

Fig. 5 Dynamics of: *1*) coenosarc pulsations in two cross sections (d1 and d2), and *2*) the rate of hydroplasmic flow 3 h after the arm of the colony was cut off. (Section of a stolon of *G. loveni* between *1* and *2* stems nearest to the stolon tip)

Discussion

There have been few publications examining the pulsatory-peristaltic transport system in hydroids (Berrill, 1949; Hale, 1960; Fulton, 1963; Marfenin, 1985a; Blackstone, 1996; Dudgeon et al., 1999; Burykin, 2010), although the alternation of hydroplasmic movement between opposite directions in the tubular coenosarc has been a known phenomenon for a long time (Cavolini 1813; Lister 1834). Berrill (1949) was the first to characterize the peculiarities of hydroplasmic currents based on close observations of fragments of *Obelia* colonies. He established that a period of regular reversal in the direction of hydroplasmic streaming is between 3 and 7 min, and noticed the lack of any designated organs generating the movement of hydroplasm within the colony. Despite the active beating of the cilia of gastrodermal cells, they cannot be the source of long-distance hydroplasmic streams. Berrill concluded that hydroplasmic flows are caused by pulsations of the coenosarc, primarily in its distal segments. Since the clipping of coenosarc tips led to immediate suspension of hydroplasmic flows toward the tips from the body of the colony, he suggested that expansion of the coenosarc that pulls hydroplasm into the growing tip is the key mechanism of hydroplasmic transport. A similar conclusion was made in further research into the osmotic nature of pulsations (Schierwater et al., 1992). Authors found that polyp contractions, stolonal contractions and gastrovascular flow were only weakly correlated on a local scale. Nevertheless, the active role of the contraction phase in the generation of HFs becomes obvious when video recordings are played back in the fast-forward mode, as well as in prolonged visual observation (Karlsen & Marfenin, 1984; Marfenin, 1985a; Burykin, 2008). Summing up his findings on *Clytia johnstoni*, Hale (1960) argued that pulsations of the regions adjacent to the growing tips of the coenosarc are the main force behind hydroplasmic movements; however, he did not elaborate on the way separate currents caused by pulsations of different growing tips interact with each other and why strong and extensive integral currents are formed as a result. Fulton (1963) conducted his research on *Cordylophora sp.*, which led him to assume that hydroplasmic movements are caused by peristaltic waves of contraction and expansion of the coenosarc, which originate in the hypostome of the hydranths. Such interpretation of the mechanism of food transport in the hydroplasm of the colony still overlooks how the peristaltic waves generated by numerous hydranths interact with each other after they meet in the tube of the stolon. Both authors worked with very small colonies.

Table 1 Key analytical parameters of coenosarc pulsations and hydroplasmic flows in a colony of *G. loveni* before and after a stolon arm was cut off from the colony ($T = 16$°C)

Episode no. Stage: before and after isolation of an "arm" of the colony (a stolon with stems)	42 Before isolation	44 Immediately after isolation	46 3 h after isolation	50 in 24 h after isolation
Time-lapse video recording period	11:55–15:16	16:19–18:44	18:46–20:51	18:47–22:30
Change in coenosarc lumen diameter (d)				
1. Average coenosarc lumen diameter at d1, μm (±SD)	78.97 ± 15.99	77.43 ± 7.30	84.05 ± 8.88	82.24 ± 4.97
2. Average coenosarc lumen diameter at d2, μm (±SD)	72.07 ± 16.92	76.46 ± 9.13	71.79 ± 8.07	78.93 ± 10.28
3. Maximum coenosarc lumen diameter at d1, μm (±SD)	135.0	97.4	103.0	100.2
4. Maximum coenosarc lumen diameter at d2, μm (±SD)	136.4	98.1	98.8	99.5
5. Coenosarc pulsations period at d1, min (±SD)	13.93 ± 3.53	13.50 ± 0.99	12.79 ± 1.13	11.58 ± 6.45
6. Coenosarc pulsations period at d2, min (±SD)	13.51 ± 3.68	13.41 ± 1.09	12.75 ± 0.76	8.20 ± 3.47
Hydroplasmic flows (HF)				
7. Resting phase—Percentage of registered absences of HF (%)	32.2	13.8	10.6	14.3
8. Percentage of HF detections towards the tip of the stolon (%)	23.7	55.9	53.7	29.0
9. Percentage of HF detections away from the tip of the stolon (%)	44.2	30.3	35.8	56.8
10. Amplitude of HF towards the tip of the stolon (±SD)	23.1 ± 16.0	60.9 ± 16.4	57.2 ± 16.4	74.2 ± 19.9
11. Amplitude of HF away from the tip of the stolon (±SD)	25.2 ± 7.0	65.4 ± 22.5	87.6 ± 15.1	68.4 ± 11.9
12. Maximum rate of HF towards the tip of the stolon ($\mu m*s^{-1}$)	55.7	83.5	79.3	116.9
13. Maximum rate of HF away from the tip of the stolon ($\mu m*s^{-1}$)	43.8	94.7	117.6	114.1
14. Period of HF towards the tip of stolon, min (±SD)	13.72 ± 4.61	12.99 ± 2.84	12.83 ± 1.00	14.75 ± 1.03
15. Period of HF away from the tip of the stolon, min (±SD)	13.59 ± 2.30	12.34 ± 3.29	12.85 ± 0.64	14.79 ± 1.86
Correlations				
16. Pearson's CC between d1 and d2, $P < 0.005$	0.892	0.718	0.788	0.216
17. Pearson's CC between d1 and HF rate, $P < 0.005$	0.222	−0.012	0.276	0.228
18. Pearson's CC between d2 and HF rate, $P < 0.005$	0.186	0.212	0.387	0.058

Ašmantas & Venslauskas (2002) analyzed the frequency dynamics of radial pulsations in the stolons of *Podocoryne carnea* using time-lapse videos made with a custom-manufactured video scanner that enabled automated recording and processing of coenosarc pulsations data. They suggested that the cyclic behavior of stolons is determined by a network of pacemakers residing in stolon wall cells and the amount and/or state of food particles inside the stolon lumen. A hydranth beats more actively after it captures food, which affects hydroplasmic currents in such a way that hydranths that did not receive any food start beating as well and thus cause the food to be transported over a longer distance (Dudgeon & Buss, 1996; Dudgeon et al., 1999). The same effect of hydranth pulsations in the formation of the arterial HF was demonstrated by Burykin (2008; 2010).

Our research was focused on the problem of interaction between pulsators, or rather, between HFs triggered by their activity (Marfenin, 1985a). Since most hydroids have highly branched colonial bodies, the study of interaction between multiple HFs within the same closed-loop system presents a major challenge. Such interaction can be observed by scanning a colony under a microscope, but it is difficult to capture such data. To date, there have not

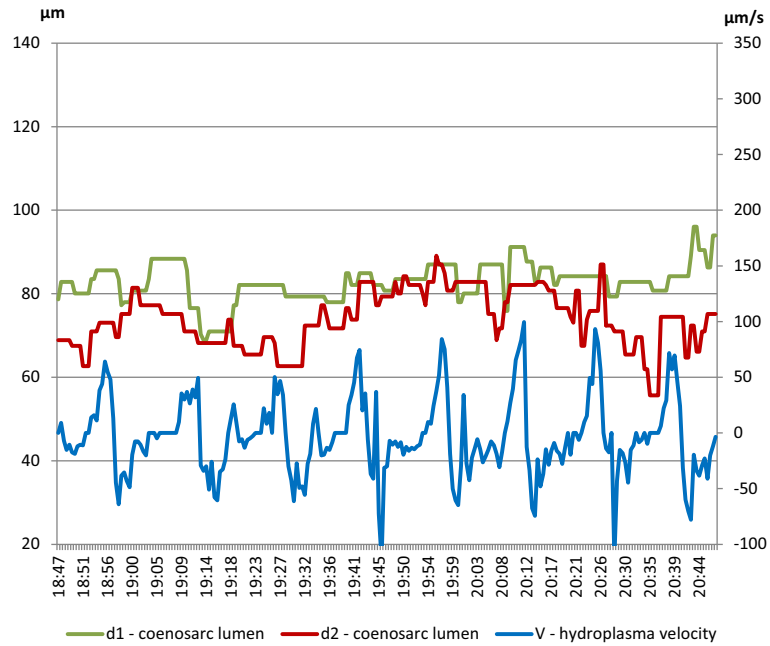

Fig. 6 Dynamics of: *1*) coenosarc pulsations in two cross sections (d1 and d2), and *2*) the rate of hydroplasmic flow 1 day after the arm of the colony was cut off. (Section of a stolon of *G. loveni* between *1* and *2* stems nearest to the stolon tip)

been any reports of successful attempts to record HFs and coenosarc pulsations in several locations of the colony at once.

Long-range HFs that regularly change direction and develop high velocities in the transport of hydroplasm were initially studied for description purposes (Karlsen & Marfenin, 1976, 1984), i.e., visual tracking of their position in space, distance traveled, and interaction between HFs in linear colonies of *Dynamena pumila* (family Sertulariidae). It turned out that along with isolated localized HFs, long-range HFs are regularly formed in the colony, first in the distal direction (toward the growing tip of the stolon), and then in the opposite direction (proximally) toward the center, i.e., the most mature region of the colony (Marfenin, 1985a). Primary distal currents (arterial HFs) are accompanied by marked luminal expansion of the coenosarc. Proximal flows originate in the «-tail» of arterial HFs, where the coenosarc lumen becomes occluded. As the arterial HF starts to abate, the distance traveled by reverse flows of hydroplasm increases, and they can be detected from the tip of the stolon almost all the way to the furthermost peripheral stem of the colony. These have been termed «compensatory flows» , since in the closed-loop system of colonial hydroids the combination of arterial and compensatory flows restores the balance of hydroplasm in different parts of the colony.

Burykin (2008, 2010) relied on visual observations to study changes in the system of HFs during the initial formation of a *D. pumila* colony after the settlement of planulae larvae, in the course of its growth, and depending on the amount of food intake. His descriptions are very detailed: he investigated the sequence of hydranth and coenosarc pulsations, as well as the direction of local HFs in small colonies consisting of one stolon with several young stems. Despite the uncoordinated pulsation activity of hydranths and various parts of the coenosarc, as well as the local HFs they produce, the overall system of hydroplasmic movements starts to act in rhythm after the colony receives food, and individual HFs are combined to form a long-range current of hydroplasm delivering food to the growing tips of the stolon. Burykin's findings confirm previous descriptions (Marfenin, 1985a, 1988) of the process of formation of the arterial HF from a series of local HFs in one or several large stems, as well as the sequence of branching of the arterial HF into the stems as it moves distally toward the stolon growing tip and reverse HFs from stems into the stolon. However, the role of self-organization in the generation of long-range HFs remains unclear.

Burykin (2008, 2010, 2013) prioritizes the essential independence of pulsators, while Marfenin (1985b, 1988, 2008) points to the priority of how they interact while maintaining independence.

Theoretically speaking, the existence of regular long-range HFs is only possible when there is mutual correlation between multiple pulsators, whether hydranths or parts of the coenosarc. One instance of such correlation was described by Ch. Wyttenbach (1973) who studied growth pulsations of the growing tips of stems. He discovered that when the growing tip contraction phase coincides with the influx of a strong HF, the growth pulsation phase shifts.

This fact was later used in the theoretical model (Marfenin, 1985a) of hydraulic integration of uncoordinated HFs. If transverse pulsations of regions of the coenosarc adjacent to the growing tips are able to respond to a strong incoming HF by delaying contraction in the same way growth pulsations do, it should result in a shifting phase of pulsations in these regions, i.e., adjustment of local pulsators to the pulsation rhythm of a strong HF. Long-term registration of HFs and lateral coenosarc pulsations in one location of the colony is sufficient to test this assumption.

Nowadays, the problem of physiological integration of the colony via rhythmic integral HFs can be studied quantitatively by recording the dynamics of particle transfer inside hydroplasm in one location in the colony, for example, every 30 s or at a different interval, instead of trying to capture the whole picture of hydroplasmic currents in the colony. This allowed us to describe the periodicity of pulsations in several species of the order *Leptothecata*, such as *D. pumila*, and later *G. loveni* and *Obelia longissima* (family Campanulariidae). Intensification of arterial HFs is observed 1–2 h after feeding. This suggests that increases in flow rates, regularity and distance traveled by HFs are caused by intensified interaction between pulsators, however, further research is needed to verify this assumption (Marfenin, 1988).

The transport system in primitive creeping colonies of *Perigonimus abyssi* and *Stauridia producta* are dominated by local HFs, which nevertheless form regular long-range HFs toward the tip of the stolon and away from it. The likelihood of their formation depends on the location of food intake. During the feeding of hydranths far removed from the growing tip of the stolon arterial HFs develop in 100% of instances, and when the nearest hydranth receives food, the occurrence of HFs is only 13% (Burykin, 2013).

Direct visual registration was initially used to record coenosarc pulsations and HFs by measuring the luminal diameter of the coenosarc and the rate of particle transfer in the gastrovascular cavity of stolons or stems under the microscope at certain time intervals (Karlsen & Marfenin, 1976, 1984; Marfenin, 1988). The same measurements can be taken using time-lapse microcinematography and video microscopy. The procedure was described in most detail by Blackstone (1996).

The proposed method for studying integral characteristics of the transport system is a modification of earlier used methods of recording HFs and coenosarc pulsations (Marfenin, 1985a, 1988; Blackstone, 1996). Instead of initial quantification of lateral coenosarc pulsations in one cross section of the stolon, we decided to measure lateral pulsations in two cross sections within the same frame. Such simple improvement opened up the possibility for registering not only stolon pulsations themselves, but also their longitudinal direction, and in some cases the independent nature of coenosarc pulsations in adjacent segments.

Another improvement to the method was to expand the system of direct indicators and calculated indices (see the list above and the table), which significantly enhanced its analytical potential and made it possible to move from description of local HFs to characterization of HF patterns in the colony as a whole.

The proposed method for video registration of coenosarc pulsations and hydroplasmic movements helps to create an comprehensive profile of the state of the pulsatory-peristaltic transport system using a combination of parameters based on three primary measurements: transverse dimensions (diameters) of the coenosarc lumen and the rate of particle transfer in the hydroplasm. As an illustration, we looked at changes in coenosarc pulsations and HF rates before and after surgical removal of a stolon arm.

Cutting off an arm of the colony immediately results in noticeable stabilization of coenosarc pulsations and acceleration of hydroplasmic flows (Figs. 3, 4, 5), observed for at least 4 h after the 'surgery'. Powerful long-range HFs may be interpreted as an indication of colony integration, since the activity of individual pulsators, whether it be the growing tips of stolons and stems, hydranths, or random combinations

thereof, cannot transport such large volumes of hydroplasm in one direction. Powerful HFs are characterized by: (1) high maximum flow rate; (2) considerable duration of unidirectional flow; and (3) maximum luminal diameter of the stolon, which pulsates within the camera's field of vision in a synchronous and strictly sequenced manner (if peristalsis occurs).

The rhythmicity and intensity of HF pulsations are still observed 24 h later, despite the desynchronization of coenosarc pulsations, which is reflected in barely defined periodicity and uncoordinated pulsations in two closely spaced segments of the coenosarc d1 and d2 (Fig. 6). Desynchronization of pulsations within the field of view of one frame is accompanied by a decline in the intensity of HF, and therefore in the degree colony integration.

The following is an example of how observations can be interpreted with the help of proposed indicators.

In our case study, isolation of the side-arm—a lateral stolon with 7 stems—led to decoupling of coenosarc radial pulsations (see d1 and d2), which can be accounted for by lower dependence of the system of HFs on the colony as a whole and increased relevance of local factors, including the growing tip of stolon itself. Before the stolon arm was cut off from the rest of the colony a high coefficient of correlation was observed between d1 and d2 pulsations (CC = 0.892, $P < 0.005$).

Within 5 h after the stolon was cut off (Figs. 4, 5), coefficient of correlation between d1 and d2 coenosarc pulsations was only marginally lower (CC = 0.718 and 0.788). 27 h after the stolon was cut off, the correlation coefficient declined considerably (CC = 0.216).

The system of hydroplasmic flows became noticeably more reactive after the severance, i.e., the share of HF registrations increased in relation to captured absences of hydroplasmic movement. It may indicate that in control measurements (taken before the stolon was cut off) a well-established system of HFs could be observed near the growing tip of the stolon, with fewer uncoordinated activity outbursts on the part of individual pulsators, as reflected in the observed transport of hydroplasm. Isolation of the stolon segment undermined the synchronization of pulsators, thus reducing the duration of hydroplasmic quiescence. Weakening of pulsation synchronicity may be explained by the disruption of established balance between pulsators as a result of surgical removal of an arm of the colony.

Maximum values of HF rate increased 1.5-fold immediately after the side-arm was severed, and doubled 27 h later, i.e., both incoming and outgoing flows (in relation to the stolon tip) increased in intensity.

Substantive data on the functioning of transport systems are not limited to the indicators listed above. Additional insights can be gathered from the fluctuations of lumen diameter from one measurement to another: $\Delta D = D(t) - D(t - 1)$.

Analysis of correlation between pulsation trends in two cross sections within the same frame where coenosarc pulsations were recorded help to detect instances of synchronous and asynchronous pulsations, including peristaltic waves of coenosarc contractions and expansions.

Coefficients of correlation between the patterns of coenosarc pulsations and the rate of hydroplasmic flow reveal either a connection between these two processes, or the absence thereof.

Finally, using the measurements of coenosarc cross sections and HF rates, the cumulative volume of hydroplasm transported over one cycle of flow pulsations can be calculated, as well as the approximate distance covered by the hydroplasmic flow as it travels throughout the colony.

As described above, this method can be used to investigate the functional dynamics of the pulsatory-peristaltic transport system and its response to external influence. First, it could become a new instrument for studying the self-organization of decentralized systems, like colonies of hydroids. Second, it offers a good possibility to use hydroids for bioindication and biotesting.

Our findings can be explained by a drop in hydrostatic pressure in the affected stolon after the lateral arm is cut off, which enhanced HF mobility. On numerous occasions we observed luminal expansion of the gastrovascular cavity after unlimited supply of food to the colony, as the cavity was filled with food particles and the mobility of HFs and distance traveled by the hydroplasm declined (Marfenin, 1988; new and previously unpublished data). Further relief of internal pressure as an effect of continued arm growth could be what causes desynchronization of pulsations.

This assumption can be tested in additional experiments using the same methodology of video registration of HFs and lateral coenosarc pulsations, followed by the use of the aforementioned set of indicators and indices to process obtained primary data.

Method tests have confirmed that videos recorded using long-term time-lapse microscopy in one location in the colony offer sufficient data for the analysis of transport system efficiency throughout the whole colony or a significant part of the colony.

So far the transport of hydroplasm and coenosarc pulsations in colonial hydroids have only been studied on a limited number of species, and there are disagreements about certain parameters, such as pulsation periods, distance traveled by HFs, the role of hydranths and coenosarc in the transport of hydroplasm.

There may be significant differences between the orders *Anthoathecata* and *Leptothecata*, in terms of hydroplasmic movement patterns. Some characterization studies of HFs and coenosarc and hydranth pulsations were carried out with *Podocoryne carnea* and *Hydractinia symbiolongicarpus* (order *Anthoathecata*). The transport function is heterogeneous in different parts of the colonies of these two species: it is more active at the periphery than in the center, more intensive in linear forms and less intensive in encrusting forms (Blackstone, 1996). When three segments of the distal part of the stolon are compared, the tendency of HF to slow down as it approaches the growing tip is observed in both *H. symbiolongicarpus* and *P. carnea* (Van Winkle & Blackstone, 1997).

Figures present an overall pattern of HF dynamics that is loosely equivalent to that recorded in *G. loveni* (order *Leptothecata*), but differs significantly in the period and maximum values of HF rates. The period of HFs is about 2 min (at T = 20.5°C) in *H. symbiolongicarpus* and *P. carnea*, and 13 min in *G. loveni* (at T = 16–17°C), with maximum HF rates reaching 300–600 μm s^{-1} and about 100 μm s^{-1}, accordingly. These differences may be explained by the significant taxonomic «distance» between the orders to which compared species belong, as well as by differences in temperature. At higher temperatures, the period of coenosarc pulsations decreases (Wyttenbach, 1968), although the average amplitude of pulsations of the stolon lumen barely changes (author's own unpublished findings).

Conclusions

Summing up the results of method tests, the following can be concluded. Considering the possible interest in colonial hydroids as biological models of decentralized self-regulation, the proposed method for studying the functioning of pulsatory-peristaltic transport system in hydroid colonies can be recommended as a tool for quantitative characterization of the state of the transport system, the distance of hydroplasmic transfer, and the interaction of pulsators that contribute to fluid propulsion, using data obtained from localized time-lapse videos recorded over long periods.

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