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Cardiovascular measurements in animals in the milligram range

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Abstract

The study of microscopic animals should be intensified because: most of the world's animal biomass consists of very small animals; life as a small animal is both qualitatively and quantitatively very different from that of a large animal; and almost all animals are very small as they begin their development. Fortunately, developing technology now allows us to make quantitative measurements in microscopic animals. This paper describes new techniques for measuring cardiovascular variables such as blood pressure, stroke volume, heart rate and cardiac output in animals weighing as little as a few mg. Non-invasive techniques such as videomicroscopy can be used for determining heart stroke volume in small animals. Impedance measurement is another non-invasive or minor invasive technique for determining rates of heart beat, gill or lung ventilation and limb movement as well as giving qualitative information on changes in blood flow. Pulsed Doppler technology can be used to obtain blood flow velocity in small vessels. Invasive techniques depend on servo-null micropressure systems that record pressure through glass microelectrodes that are implanted into the vessel or heart lumen. This allows stable pressure recordings for up to 5-6 h in animals weighing as little as a few mg. Microinjectors can be used for intravascular injections of vasoactive drugs (or blood withdrawals). Newly emerging techniques for *in vivo* cardiovascular measurements allow us to understand the function of the cardiovascular system in a larger portion of the world's animal biomass, as well as in the immature and as yet poorly understood early developmental stages of animals.

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- Blood flow
- Cardiac output

Introduction: Why study small animals?

The last decades have seen a rapid proliferation of technological advances that have changed the way that physiological measurements are made. Much attention has focused on the acquisition of physiological data once thought unattainable. Examples are regional metabolic activity of the brain and *in vivo* recordings of intracellular pH. Some of these measurements required expensive, complex techniques such as nuclear magnetic resonance (NMR) and positron electron tomography (PET). Yet, exciting advances have also been made on another, less obvious front - miniaturization of existing techniques and development of relatively inexpensive new ones for measurement of more conventional physiological variables such as heart rate, blood pressure, stroke volume and cardiac output. These technologies now allow cardiovascular physiologists, for example, to measure cardiac output and central arterial pressures in animals of a few mg body mass!

Why are some physiologists pushing the frontiers of miniaturization when so many larger animals that could be suitable as models are unstudied, and certainly present fewer technical hurdles? Setting aside the considerable appeal (to some) of meeting the technical challenge of successfully making these measurements, there are at least three reasons for the recent upsurge in papers reporting on the physiology of extremely small animals.

Most animals are small

The first compelling reason to develop techniques for studying physiological processes in small animals is simply stated - almost all of the world's animal biomass, and almost all of its animal species, consist of very small animals. An estimated 90-95% of all Metazoans are thought to be Arthropods, for example, which typically weigh a few grams at most. Increasingly, animal physiologists are turning to invertebrates (and often insects) as experimental paradigms. In part this trend is fueled by a resurgence of invertebrates for their own sake, since they are of overwhelming biological importance. However, the increasing bureaucracy and accelerating costs of carrying out research on vertebrates is causing investigators in many countries to turn towards physiological experimentation on invertebrates.

Small animals differ from large animals

A second compelling reason for studying very small animals, and thus for developing the necessary techniques, is the realization that life as a small animal is both qualitatively and quantitatively different than as a larger animal. Physiologists have appreciated for centuries the fact that metabolism, cardiac function, breathing, and a myriad of other physiological processes show scaling or "allometric" relationships, and the last decade has seen several important titles on this subject (1-4). Much emphasis has been placed on the so-called "mouse to elephant" curve, in which there are large physiological differences within mammals between a 50-g mouse and a 5000-kg elephant. While we may view these 5 log-cycles of variation in body mass as quite enormous, they are in fact similar to the body mass difference within crustaceans between say, the water flea *Daphnia* and a small shore crab. Often not fully appreciated, however, is that extremely small animals may be even more different anatomically and physiologically from their larger counterparts because of the physicochemical consequences of being so small. Figure 1 (Ref. 5) indicates how velocity, body mass, Reynolds number, inertia and viscosity of the environment interact over

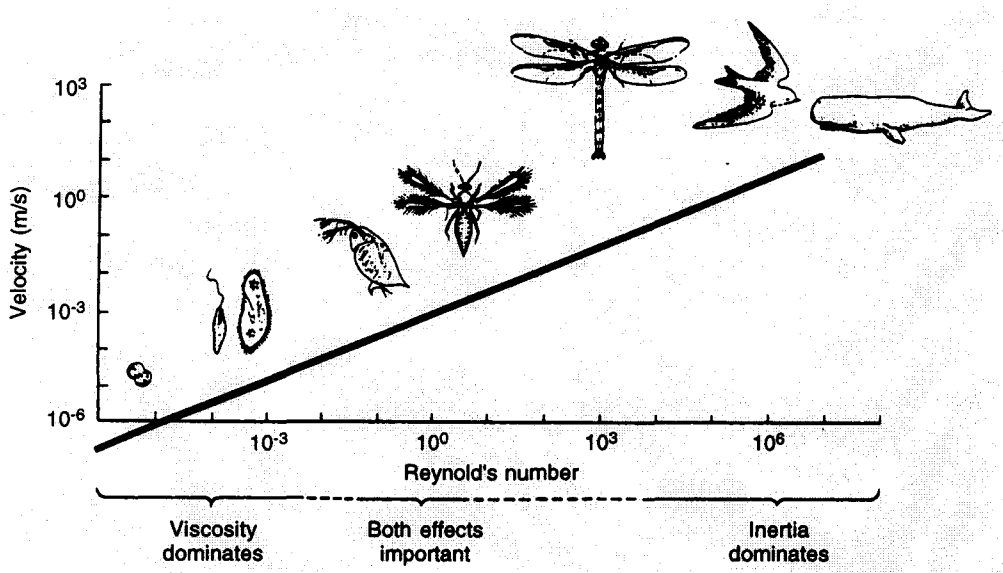

Figure 1 - Relationship between body mass, Reynold's number and velocity of locomotion of animals. In large animals inertial forces dominate locomotion, while in small animals viscosity effects dominate locomotion. (Reproduced, with permission, from Eckert et al., Ref. 5).

the span of total animal body mass. Locomotion (which of course depends upon effective gas exchange and internal cardiovascular transport) is dominated by inertia in large animals, but as body mass declines inertial effects give rise to viscosity effects at a low Reynolds number. As most recently emphasized by Koehl (6), life at a low Reynolds number does indeed constitute a strong selection pressure for morphological and physiological evolution.

Small animals grow into larger animals

A final reason for studying very small animals is expressed well by McMahon and Bonner (4), who state in the opening of the preface to their excellent book *On Size and Life*, "Once, we were smaller than we are now." Indeed, virtually all organisms grow in size as they develop. There is a burgeoning interest in developmental physiology, especially of the processes of respiration and circulation in both vertebrates and invertebrates. The study of the physiology of animals in their early stages of development is giving us considerable insight into not only the development but also the evolution of physiological systems (7,8). Structures or specific processes that we identify in adults, but to which we can ascribe no necessary function, may in fact represent the remnants of a condition that was vital for the development of small organisms. For example, the literature is replete with analyses of the blood oxygen transport properties of Amazonian fishes, both air breathers and water breathers. Yet, there is still no unequivocal interpretation or clear pattern of adaptation in blood properties. Perhaps the study of the immature, larval forms of these species in environmental conditions appropriate for that stage of development (which may be very different from what the adults experience) will show that a particular set of blood oxygen transport characteristics may be crucial to the survival and continued growth of the larvae, but of lesser importance in adult animals. If the field of developmental physiology is to grow, however, then so too must the technology allowing measurements in very small animals.

Given the importance of making physiological measurements in very small animals, how are they to be achieved? An extensive survey of all available technology is well beyond the scope of this paper.

What we wish to do is show how cardiovascular measurements in particular are being achieved, with the hope of encouraging and stimulating creative efforts in the readers at measuring other types of physiological parameters. Table 1 lists the addresses of some manufacturers whose products have been used in the experiments described below.

Cardiovascular measurements in small animals

In this paper we will consider two sets of recent advances in cardiovascular measurement - non-invasive techniques and invasive (or minimally invasive) techniques. The relative pros and cons of non-invasive *vs* invasive approaches in physiological

Table 1 - Sources of specialized equipment.

Application	Technique/ equipment	Manufacturer	Address
Rates of movement (heart beat, gill or lung ventilation etc.)	Impedance convertor	UFI	545 Main Street Moro Bay California, USA
Blood flow	Pulsed Doppler techniques	The University of Iowa, Bioengineering	56 M.R.F. Iowa City, IA 52242, USA Fax: (319)335-8644
Blood flow	Doppler crystals	TMI	Iowa City, IA, USA Fax: (319)338-0836
Stroke volume	Videomicroscopy	Optimas Bioscan	Bioscan Inc. 170 West Dayton Suite 204 Edmonds, WA 98020, USA
Blood pressure	Dual servo-null micropressure system 900A	WPI	175 Sarasota Center Boulevard, Sarasota, FL 34240-9258, USA Fax: (813)377-5428
Microinjection	Nanoliterinjector	WPI	"
"	Pneumatic picopump PV830	WPI	"
"	Picoinjector PL100	Medical Systems Corp.	One Plaza Road Greenvale, NY 11548, USA Fax: (516)621-8503
"	Transjector 5246	Eppendorf	Hinz GmbH 22331 Hamburg, Germany, Fax: (49)4053801556

studies have been previously discussed in a general sense (9), and we assume that the reader is aware of the advantages and pitfalls of each approach.

Non-invasive techniques

Video microscopy

The use of photographic cine film images to analyze physiological events dates backs many decades. However, the advent of high resolution, low light video cameras and computer-assisted analytical procedures has brought video microscopy to the forefront of cardiovascular investigation of small animals (Figure 2). In theory, the procedures are quite simple. A black and white or color video camera is mounted on a dissecting microscope. Video recordings are then made of magnified images of the beating heart (or hearts) of the animal. The minimum size of the animal really depends on the quality of the optics of the available microscope and the lighting. Heart rate can be determined from the image by looking at heart movement or the pulsing blood or hemolymph passing through central or peripheral vessels. If the outline of the heart can be clearly visualized, then stroke volume can be determined from the rhythmic changes in heart volume, and ultimately cardiac output can also be calculated, as described below. In practice, of course, making such measurements requires careful preparation. Two aspects are critical - adequately immobilizing the animal and choosing the appropriate lighting.

1. *Immobilizing the animal for observation.* An animal's heart may well fill the field of view of the microscope at higher magnifications. Because many measurements are based on relatively subtle changes in heart dimension, it is critical that the animal be completely immobilized while being videotaped. Anesthetics are one option, but they may also interfere with the physiological process of interest. MS222, a common anesthetic for fishes and amphibians,

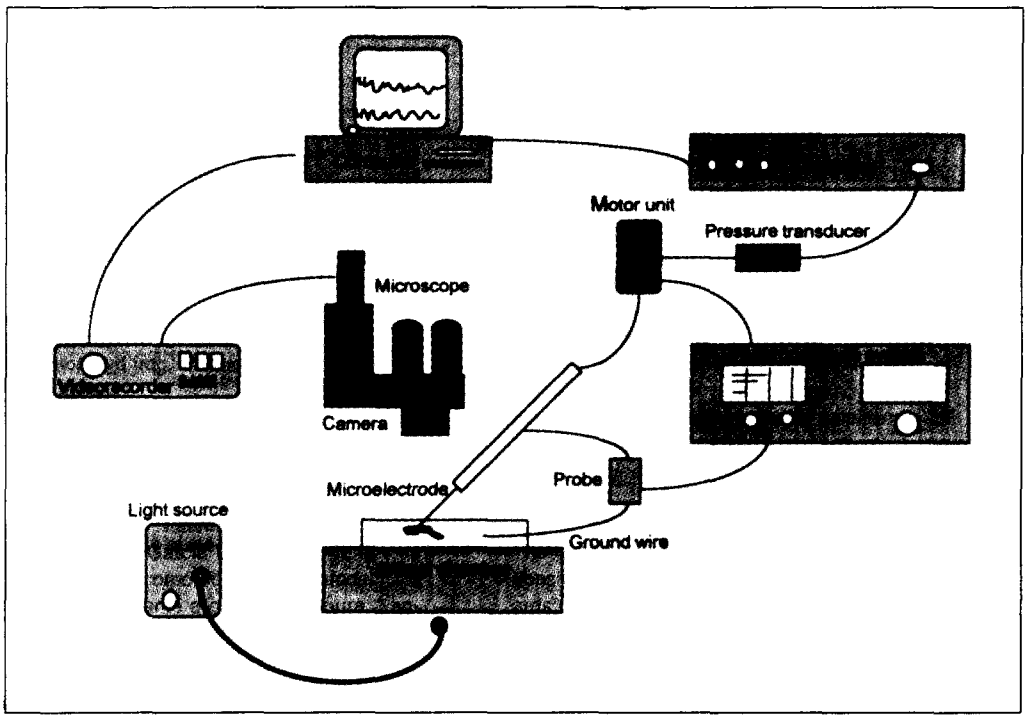

Figure 2 - Typical arrangement of a microscopy system for *in vivo* measurement of cardiovascular variables including heart rate, stroke volume, cardiac output, and central arterial and ventricular pressures. Video images of the beating heart made through a dissecting microscope are stored in a data acquisition system for subsequent image analysis. Microelectrodes inserted into the circulation are used to record blood pressures, also stored by a data acquisition system. See text for additional details.

is a sympathetic cervical stimulant, for example. For extremely immature animals with as yet poorly developed nervous systems, anesthetics may not be sufficient because some body movement may result from spontaneous contractions of developing myotomes that occur independently of neural motor stimulation.

If anesthesia is not appropriate, then physical restraint is an option. Tiny pins can be used to "fence in" an animal on the microscope stage. An alternative to pins is cactus spines, which come in a wide range of bizarre and potentially very useful shapes resembling microscopic spears, hooks and pins. Very fine nylon mesh (such as that in nylon stockings) can be used along with pins or spines to "net" or entrap the non-anesthetized animal, yet still allow free movement of air or water over the animal's surface. Another technique for immobilizing aquatic species involves trapping the animal in solid agar. Microbiological supply houses sell an agar that melts at 26-27°C. Such an agar solution is heated until it just liquefies, and the intact animal to be studied is placed within it. The agar is then cooled, and the animal becomes trapped within a solid agar matrix. The block of agar containing the animal is then quickly cut out and transferred to the microscope stage. Agar has approximately the same gas diffusion properties as unstirred water, so simple diffusion of gas across a thin agar boundary will be adequate for animals that use their general body surface for respiration. This necessitates keeping the surrounding agar layer as thin as possible while still physically restraining the animal. In the case of larval fish or amphibians with specialized gas exchange organs, fine surgical tools can be used to sculpt the agar away from around the mouth and head to allow spontaneous or artificial gill ventilation, while still keeping the body generally immobilized. Agar can also be selectively removed from the area of the body surface by the heart, for example, to allow surgical intervention. The partial pressure gradients across the agar must also be kept as large as possible by surrounding the block in oxygen-enriched gas.

2. *Illumination*. Effective illumination of the animal is necessary for optimal video images. Commercially available fiber optic illuminators with flexible light bundles are very useful in this regard. Individual light shafts of 1 mm diameter or less can also be used to provide pinpoint illumination of particular body regions. Fiber optic illumination has the advantage of not generating heat in the immediate vicinity of the illuminated tissue, an important consideration in very small animals with low thermal capacitance.

In many applications, reflected light coming off moist, curved surfaces is problematic, because it obscures details of underlying images. Substage illumination, in which light is projected from under the animal towards the objectives, often eliminates this problem (Figure 2). It can actually accentuate internal structures (heart, gills, major blood vessels). Substage illumination can be easily achieved by placing the animal in a glass-bottomed container, and then using a curved fiber optic light shaft or an angled mirror to direct light in the appropriate direction.

3. *Analysis of video images*. Once appropriate video images have been captured on videotape, they can be subjected to a wide range of analyses. Simplest to extract is heart rate from the rhythmic changes in heart shape and/or size. Because very small animals often have heart rates that exceed 1-2 Hz, slow motion capability for viewing videotapes is very useful for determining heart rate accurately. A videotape recorder with digital freeze frame capabilities will greatly facilitate analysis of heart beat and shape changes by eliminating the flickering of frozen images often produced by

conventional domestic recorders. Frame-by-frame analysis can be used to determine both qualitative and quantitative aspects of heart filling and emptying. Recently, video microscopy of the beating heart has been used to calculate stroke volume in larvae of the anuran amphibian *Xenopus laevis*, (10-12; Fritsche R and Burggren W, unpublished results). Preliminary experiments involving videotaping of the beating heart from several angles are used to determine the three dimensional shape of the heart. In the case of *Xenopus laevis*, the heart is quite accurately portrayed as a prolate spheroid, whose volume is given by the formula $V = 4/3\pi ab^2$, where a and b are half of the measured long and short axes, respectively, of the ventricle. With this information on heart shape, total heart volume can be calculated either by measuring linear dimensions of the heart (13), or by calculating its surface area in a two-dimensional analysis and from this value deriving heart volume (Fritsche R and Burggren W, unpublished results). These data can now be readily derived from video images either by using a digitizer or by any of the several commercially available video analysis software packages produced by Optimus, Jandel and other software companies (see Table 1). By advancing frame-by-frame through the video images of a beating heart, it is quite easy to identify end-diastolic ventricle volume (largest volume) and end-systolic ventricle volume (smallest volume). Assuming that the contracting and relaxing ventricle walls undergo shape and thickness changes but no significant change in wall volume (i.e., that muscle is incompressible), the difference between the end-diastolic and end-systolic volume of the ventricle is equivalent to stroke volume of the heart. Knowing stroke volume (SV) and heart rate (f_H), cardiac output (CO) can be readily calculated: $CO = SV \times f_H$.

Impedance measurements of cardiac activity and blood flow

The conversion of changes in biological impedance into a proportional DC voltage by use of an "impedance converter" (sometimes called an "impedance pneumograph") has also proven to be another relatively non-invasive or minor invasive method for measuring physiological events in very small animals. Rates of heart beat, gill or lung ventilation, and limb or other appendage movement, as well as qualitative indications of changes in the flow of blood, water or air, can be determined in animals of less than 1 mg with such devices. Impedance convertors can be acquired commercially from UFI (Table 1) at very reasonable cost.

The theory of impedance conversion is as follows. Basically, any change in the shape, volume or composition of biological tissue will be accompanied by minute but detectable changes in that tissue's impedance (a "resistance" to an oscillating current with capacitance components). Every time a heart beats, a buccal cavity compresses, or a surge of blood pulses through a blood vessel, the impedance of the tissues in the immediate vicinity of that tissue motion will be altered. Although the way in which the impedance signal is acquired will vary from preparation to preparation, the basic arrangement consists of implanting copper or silver wire electrodes in the vicinity of the region of interest. The electrode wire, which can be as small as a few tenths of a mm, must be insulated. The insulation is removed (by burning or scraping) from the last 0.5-1.0 mm of the implanted end of the wire. The uninsulated electrode tips can then be implanted in tissue (or placed in saline) on either side of an animal's heart, or they can be placed near an expanding and contracting buccal or opercular cavity or lung. Any change in

impedance measured between the electrode tips will be converted into a proportional DC signal that can be recorded on a chart recorder or data logger. Figure 3 shows how heart rate and/or gill ventilation can be detected in a very small aquatic organism such as *Daphnia* placed within an irrigated capillary tube. Note that, as long as the surrounding medium has conductive properties, it is not necessary to penetrate the animal's body with the electrodes. Clearer signals are achieved if the electrodes are implanted within the body tissue, however. One tremendous advantage of impedance conversion over conventional electrocardiography is that, unlike in the recording of an ECG where a Faraday cage or other precautions against electrical noise must be employed, no special grounding or shielding is necessary to detect impedance signals.

Generally, impedance signals are most useful in determining a pulsatile waveform from which rates are counted. However, the amplitude of the waveform derived from an impedance signal measured across a blood vessel, for example, can provide semi-quantitative information on changes in flow. Although measured on a large turtle, the records in Figure 4 (Ref. 14) of pulmonary blood flow measured simultaneously with an electromagnetic flow meter around the pulmonary artery and impedance electrodes placed in the lung walls show the very close relationship between blood velocity/flow and the impedance signal of blood moving in the vessels (14). Thus, for example, it is possible to generate dose-response curves for pharmacological treatments of the cardiovascular system based on arbitrary, non-calibrated units of blood flow.

Pulsed Doppler measurement of blood flow

Pulsed Doppler technology has largely overtaken electromagnetic techniques for blood flow measurement through vessels. Major vendors include Titroinics Medical Instruments, Crystal Biotech, Transonics

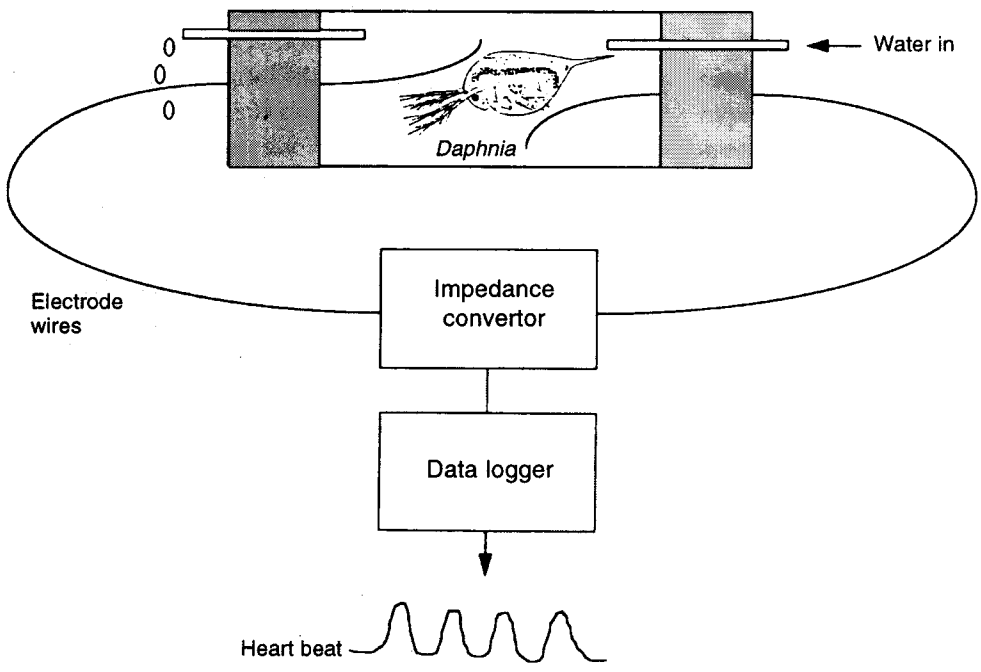

Figure 3 - Heart rate can be measured in intact water fleas (*Daphnia*) using an impedance conversion system. Fine wire electrodes in the vicinity of the animal are used to record impedance changes caused by movement of the heart or appendage movement.

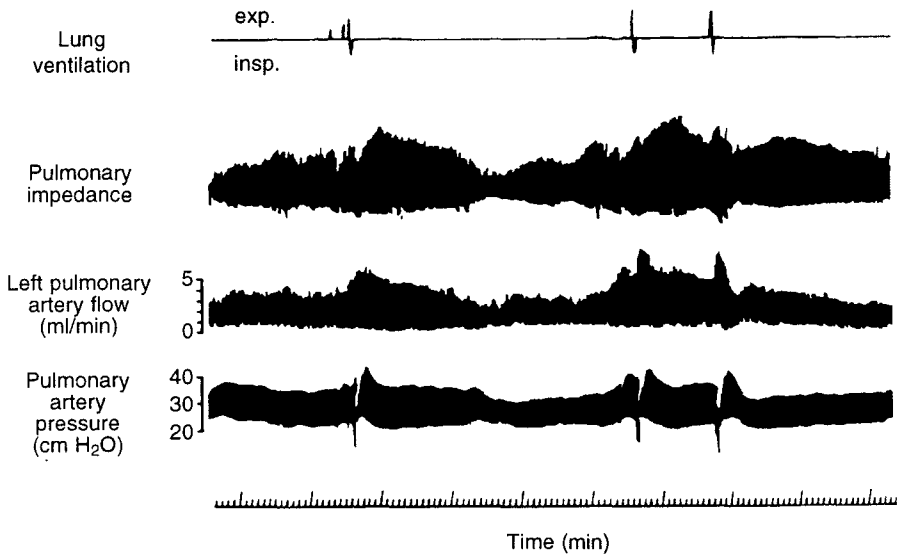

Figure 4 - Pulmonary blood flow and arterial pressure measured during voluntary intermittent breathing in the turtle *Chelodina longicollis*. Note the close correspondence between pulmonary blood flow measured qualitatively by impedance using copper wire electrodes implanted in lung tissue and measured quantitatively by an electromagnetic flow transducer placed around the left pulmonary artery. (Reproduced, with permission, from Burggren et al., Ref. 14).

Systems and Iowa Doppler Products (see Table 1). Essentially, the blood flow transducer that is placed adjacent to the blood vessel of interest consists of a piezoelectric crystal in the form of a 0.2-0.4-mm thick disk about 1 mm in diameter. The crystal is given an exciting current pulse through two connected wires, causing it to vibrate and give off ultrasonic energy. The ultrasound is reflected from structures in the immediate surroundings. The crystal, which then temporarily enters a non-excited phase, becomes vibrated by the reflected energy, and in response generates a current in the wires. By alternating between acting as a "speaker" and a "microphone", a piezoelectric crystal connected to the appropriate oscillators and signal conditioners can be used to detect the reflected sound from moving objects (typically blood cells in plasma). The electronics calculate the Doppler shift from these signals, and then use this information to generate a DC current proportional to object velocity (essentially, blood velocity). Blood flow can then be calculated knowing the exact angle of the crystal relative to the direction of blood flow, the nature of the energizing current, and the diameter of the blood vessel.

Although pulsed Doppler transducers are much more compact than electromagnetic transducers, they are still enormous compared to the vessels of many microscopic animals in which we might wish to measure blood flow. However, a "mini-transducer" can be made by mounting the bare crystal onto a shaft held in a micromanipulator. By maneuvering the crystal into position near the body surface of an animal, semi-qualitative signals of blood flow through major vessels can be achieved. In fact, as shown in Figure 5, we have used bare crystals to monitor blood movement through the single capillary loop of an external gill filament in a 23-mg larva of the salamander *Ambystoma maculatum*.

Invasive techniques

Most cardiovascular measurements in very small animals have focused to date on changes in heart rate. In part, this may be due to the fact that it is relatively easy to determine heart rate (see above). Heart rate changes with development have been investigated in invertebrates as well as all groups of vertebrates. However, much less

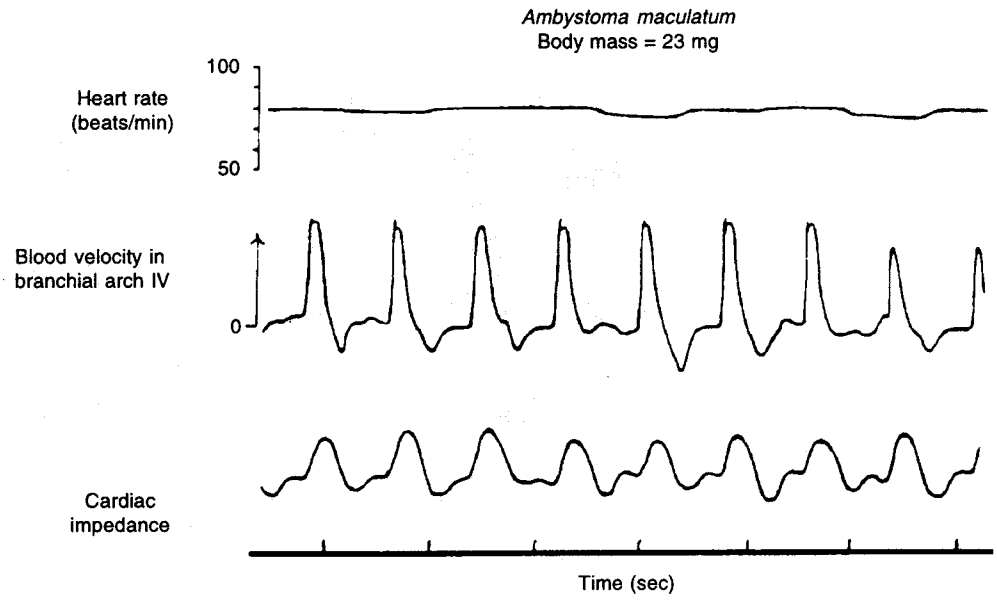

Figure 5 - Heart rate, blood velocity in branchial arch IV, and cardiac impedance measured in an intact, free swimming 23-mg larva of the salamander *Ambystoma maculatum*. Blood velocity was measured with a bare pulsed Doppler crystal mounted in a micromanipulator and held approximately 1 mm from the external gill arch. Cardiac impedance was measured with fine copper wire electrodes placed in the water near the body wall overlaying the heart. See text for additional details of methodology.

information exists on changes in overall cardiovascular performance (blood pressure, stroke volume, cardiac output and peripheral resistance as well as heart rate) in very small animals. Although micropressure systems have been commercially available for more than two decades, it was not until fairly recently that papers on total hemodynamic changes with development, for example, have come out (12,13,15,16; Fritsche R and Burggren W, unpublished results).

Continuous heart rate recordings are easily obtained from the pulsatile blood pressure signal via a tachograph unit. Together with videomicroscopy techniques for obtaining stroke volume, both cardiac output and total peripheral resistance can be calculated. A two-channel micropressure system can be used to measure pressure in two vessels simultaneously, permitting calculations of resistance changes over different vascular beds. Micropressure systems can also be used to measure pressure in *in vitro* or *in situ* perfused vascular beds.

Just as required when determining stroke volume from video-filmed cardiac images, *in vivo* recordings of blood pressure require both proper immobilization of the animal and appropriate lighting (see above).

Servo-null micropressure techniques for blood pressure measurements

1. *Principle.* There are basically two commercially available dual servo-null micropressure systems: a two-channel model from Instrumentation for Physiology and Medicine (IPM) and a one-channel model from World Precision Instruments (Table 1). Both systems record pressure through microelectrodes pulled from glass capillaries (1-mm outer diameter) which are filled with at least a 0.5 M NaCl or KCl solution (Figure 2). The glass capillaries are pulled using a vertical or horizontal puller to an ideal tip length of 1-1.5 mm and a diameter of 5-10 μm.

The principle of operation is quite simple. The electrode, mounted in a micromanipulator, is inserted into the vessel or heart chamber of interest. The higher pressure inside the vessel will cause the interface between the plasma and the NaCl solution to move inwards in the electrode.

This results in increased resistance inside the electrode since the resistance of plasma is higher than that of the NaCl solution. The change in resistance is measured and is proportional to the change in blood pressure. A motor-driven pump associated with the micropressure system produces a pressure in the microelectrode that just offsets the pressure in the vessel. This opposing pressure keeps the interface at a stationary position. Therefore, it is called a dual servo-null system. The pressure generated in the microelectrode system is then monitored with a conventional pressure transducer (Figure 2). Frequency responses are typically high enough to record pressures accurately in lower vertebrates with heart rates up to about 200 beats/min.

2. *Preparation of the electrodes.* One of the most important aspects when measuring micropressure is microelectrode preparation. What constitutes an optimally configured microelectrode? First, the glass capillary should be pulled into an electrode with as short a tip length as possible (about 1-1.5 mm). A long tip has a too high resistance and is also too flexible, both features leading to unstable pressure recordings. Furthermore, it is more difficult to penetrate blood vessel walls or other tissues with a long flexible electrode tip. The tip length and diameter are changed by adjusting the settings for heat (to melt the glass), delay (to shut off heat before pulling) and tension (for the pull) on the pipette puller. Low heat, short delay and high tension result in short tips and consequently high heat, long delay and low tension result in long tips. It takes patience and much practice to get the right settings for a perfect electrode, because it is a highly empirical process.

Filling the electrodes is the second crucial step. The electrodes should be filled with NaCl or KCl solution at a concentration between 0.5 and 3.0 M. The smaller the tip of the microelectrode, the higher is the required molarity of the filling solution. Boiling the solution for approximately 15 min before use helps eliminate the formation of air bubbles inside the electrodes. Even the smallest, almost invisible bubble inside the electrode will severely interfere with pressure measurements. Large amounts of a dye such as regular food coloring added to the NaCl solution help visualization under the microscope of the very fine electrode tip. The electrodes are best filled via a pulled PE10 tubing on a syringe inserted into the non-tapered end (back) of the electrode. The very tip of the electrode is filled by suspending the electrode tip down in a vacuum jar containing the NaCl or KCl filling solution. Vacuum should be applied for three periods of 5 min each just before the electrodes are to be used to avoid crystallization of the filling solution inside the electrode tip.

3. *Insertion of the electrodes.* The microelectrodes are placed in an electrode holder which in turn is secured into a micromanipulator (Figure 2). It is very important that the whole experimental set-up rests on a stable table. Sometimes better stability is achieved by placing the equipment on a heavy marble or metal table (the latter useful for micromanipulators with magnetic bases). Before inserting the electrode into the vessel, the body wall, any mesenteric tissue, and pericardium must be carefully dissected away. Otherwise, the electrode tip is easily plugged by passing through these tissues, or the tip is broken during insertion. The micromanipulator is used to maneuver the electrode immediately above the desired place of insertion. Penetration is most easily made by advancing the micromanipulator forward in a small but sudden movement. Once inside the vessel pulsatile pressure should be recorded. Very fine back and forth movements of the electrode without change in the recorded pressure will indicate that the electrode is properly lo-

cated in the lumen of the vessel.

When recording from a contractile structure such as the ventricle, conus arteriosus, bulbus arteriosus, etc., tissue movements eventually cause displacement of the electrode. This makes continuous recordings from these structures very difficult. For long-term recordings of blood pressure, placement of the electrodes in non-contractile vessels are recommended.

Figure 6 shows continuous recordings of blood pressure in the non-contractile truncus of *Xenopus laevis* at different stages of development. In fact, successful continuous recordings of up to 5-6 h have been made from the truncus arteriosus in larval *Xenopus laevis* weighing as little as 1.2 mg (Fritsche R and Burggren W, unpublished results).

Micropressure systems can also be used

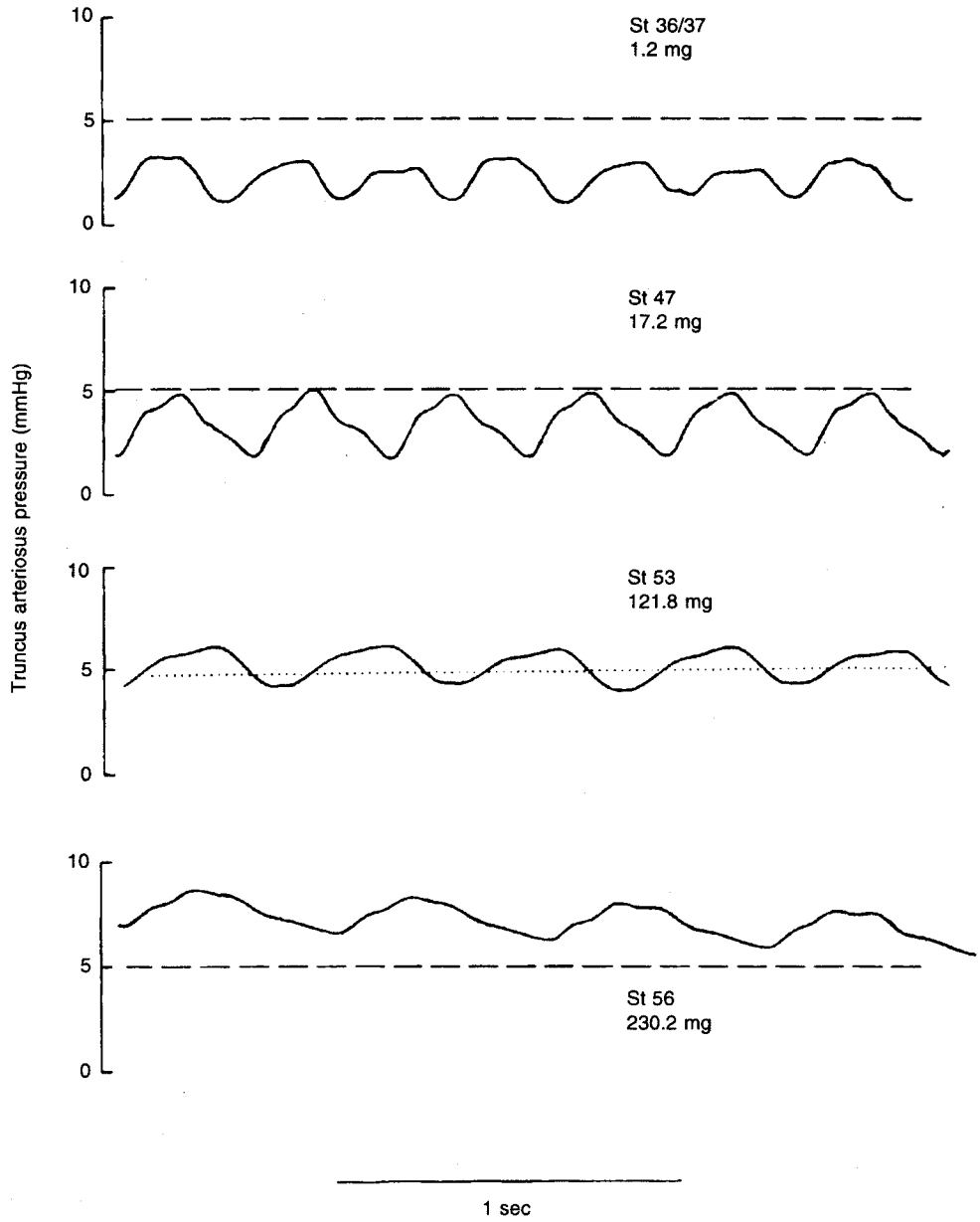

Figure 6 - Continuous recordings of blood pressure in the non-contractile truncus arteriosus of *Xenopus laevis* at four different NF developmental stages.

to measure pressures *in vitro* or *in situ* in perfused organ preparations, including gills of fishes, amphibians or crustaceans. Some tissues such as the gills of crabs that have a chitinized cuticle may require that a sharpened "stiletto" be first used to create a minuscule hole into which the microelectrode tip is then inserted. Such puncturing instruments can be made of drawn down solid glass rods, or electrically etched tungsten wire.

Buccal, opercular or branchial chamber pressures in small aquatic animals such as larval crayfish can also be measured with micropressure systems (Reiber C, unpublished results).

4. *Calibration*. Careful calibration must be performed, because the pressures found in at least developing circulations at best may be only a few mmHg above ambient. Zero pressure can be determined before and after each experiment by placing the electrode under the surface of the saline (and at the level of the heart or vessel for systems like WPI that have an air-filled pressure transducer). It is important to check for zero calibration both before and after the experiment since zero can change during especially long-term recordings. The precision of the micropressure system is verified by calibrating the system's pressure transducer against a static column of water or mercury, as described in the owner's manual.

Microinjection

The micropressure techniques described above allow us to perform accurate cardiovascular measurements in animals weighing as little as 1 or 2 mg. We know that the cardiovascular system in adult animals is under continuous influence from autonomic nerves and circulating hormones and other chemical substances. Pharmacological studies in large animals often involve directly injecting different agonists and antagonists into the cardiovascular system. In small animals, however, these types of experiments can become a big challenge.

1. *Microinjectors*. There are several microinjectors currently available (Table 1). WPI, for example, carries several models that can be used for injection (or withdrawal) of fluids such as electrolytes, acids, bases, dyes and drugs. The volumes of injection (or withdrawal) range from picoliters to milliliters. Fluid is delivered through glass capillaries that are prepared in a similar way as described for the pressure recordings.

2. *Pharmacology*. When doing intravascular injections in small animals the volume has to be carefully calculated based on the body weight and assumed blood volume to avoid volume loading the animal. Unfortunately, blood volume is rarely known, and must be assumed based on interpolation of data from larger animals. Pharmacological cardiovascular studies of small, developing animals have used topological administration of drugs onto the heart in *in situ* and *in vitro* cardiovascular preparations (17-19). Topological injections have the disadvantage that differences in heart muscle thickness between individuals may result in different diffusion distances for the applied substance and hence different magnitudes of physiological responses. Intravascular injections more directly provide qualitative information from which it becomes possible to construct accurate dose-response curves for different transmitters and different tissues. Figure 7 shows the effect of an intraarterial injection of epinephrine (100 nmol/kg) on blood pressure and heart rate in a larval *Xenopus laevis* (10 mg). Defining the development of cardiovascular control mechanisms is very important also in understanding the complex mature cardiovascular system.

3. *Volume load/depletion*. Microinjectors

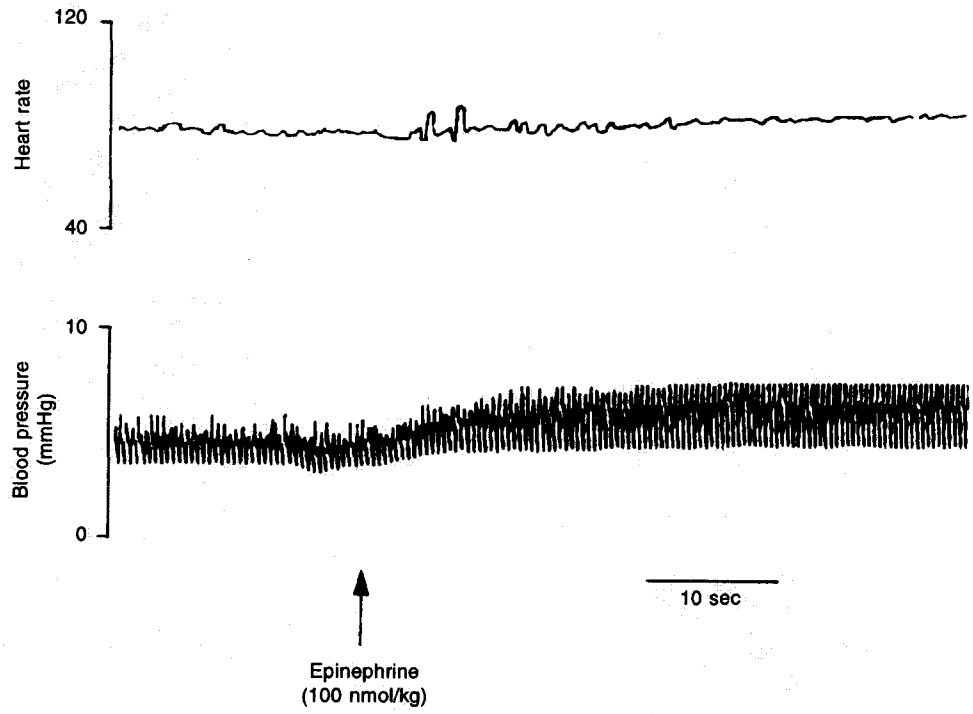

Figure 7 - Cardiovascular response produced by an intraarterial injection of adrenaline (100 nmol/kg) on blood pressure and heart rate in a larval *Xenopus laevis* weighing 10 mg. The injection was made via a microelectrode directly into a central artery. See text for additional details of methodology.

can be used for a variety of experiments apart from pure pharmacological studies. Frank-Starling mechanisms can be studied by injecting Ringer's solution into the circulation (volume loading the animal) and videotaping the heart to determine stroke volume changes. Similarly, injectors that can be switched to withdraw can be used to withdraw blood or other body fluids from the animal to alter cardiovascular performance.

Concluding remarks

Both the technology and the curiosity required to study cardiovascular physiology in animals in the milligram range are burgeoning, and we are seeing increasing numbers of laboratories that now routinely measure some aspect of cardiovascular performance "under the microscope". In some instances the organisms being studied are innately small, even as adults (e.g. *Daphnia*). In other instances, investigators have a developmental focus, and are studying the embryos/larvae of animals that have been well studied as adults using traditional technologies (e.g. the domestic chicken, *Xenopus*). Yet, we are only just beginning to understand the physiological processes occurring in microscopic organisms. Major challenges remain to be met. Cardiovascular physiology is of course intimately linked with respiratory physiology in the form of gas exchange and blood gas transport. Microelectrodes for *in vivo* measurement of P_{O_2}, P_{CO_2} and pH have existed for years, but have not been applied to microscopic organisms (as opposed to microscopic vessels in large organisms) in any systematic fashion. Understanding how the cardiovascular system of microscopic animals changes in response to respiratory adjustments, and *vice versa*, by combining microtechniques for cardiovascular and blood gas measurement, will be one of the major challenges of the next decade.

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