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Chapter X

Cardiovascular System

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X.1 TOPIC BACKGROUND

The typical fish cardiovascular system is comprised of 4 major parts: a heart, blood vessels, blood, and control systems. Most water-breathing fishes have a closed, single-circuit circulatory system where blood flows in a loop from the heart through the gills, to the peripheral tissues, and back to the heart (Figure X.1). Air-breathing fishes have more complicated circulatory systems (see Burggren et al. 1997). For a comprehensive review of the form, function and control of the fish vasculature and heart, see Sandblom and Gräns (2017) and Farrell and Smith (2017).

The cardiovascular system transports oxygen, carbon dioxide, nutrients, wastes, immune factors, and hormones around the body. As such, it plays an essential role in supporting physiological processes such as digestion, locomotion, reproduction, osmoregulation and immune function (Farrell 2011; Gamperl and Shiels 2013).

This chapter outlines some of the most common techniques used to assess cardiovascular function in fishes. We specifically address the technical aspects of using vascular casts to assess blood vessel morphology, single and repeated blood sampling, the major hematological techniques, and in vivo measurements of heart rate, blood pressure and blood flow. We do not address any histological or microscopy techniques to evaluate morphology, cellular techniques to measure electrical excitability of the cardiomyocytes, or in vitro or isolated perfused heart techniques to measure cardiac function. For these methods, see Farrell and Smith (2017), Shiels (2017) and Vornanen (2017).

X.2 METHODS

X.2.1 Technique: Vascular Corrosion Casts

To reveal the vascular anatomy of fishes, researchers can make casts of the blood vessels perfusing the tissue of interest (e.g., coronary circulation supporting the heart). There are four main casting compounds that can be used depending on the desired data; Mercox (Ladd Research, 83 Holly Ct, Williston, Vermont 05495, United States), Microfil (Flowtech 834 Carv-

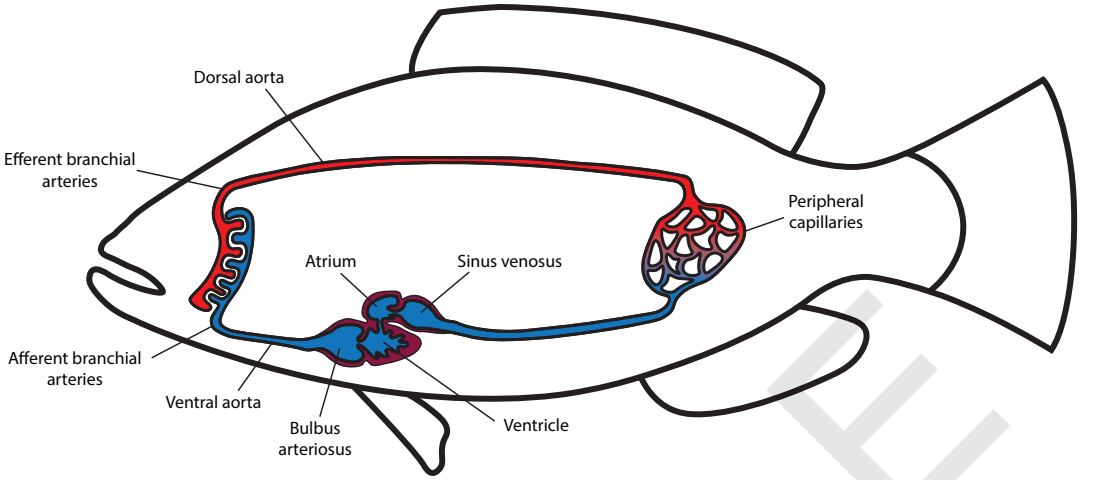

Figure X.1. Schematic diagram of the circulatory system of a 'typical' water-breathing teleost. Blood returns to the heart via the sinus venosus, and is pumped through the atrium, ventricle, bulbus arteriosus, ventral aorta, and afferent branchial gill arteries to the gill lamellae for gas exchange. Blood leaves the gill lamellae via the efferent branchial arteries and enters the dorsal aorta for distribution to the peripheral tissue capillary beds. Graphical illustration by Paul Parsons.

er, Massachusetts 02330–0834 United States), VasQtec (VasQtech, Seefeldstrasse 295, 8008 Zürich, Switzerland), and Batson's #17 (Polysciences, Inc. 400 Valley Road, Warrington, Pennsylvania 18976). Regardless of the casting material used, visualizing or analyzing tissue vasculature can be a key component to help understand the physiology and functional morphology of organs and organisms. With all compounds, the preliminary steps for tissue preparation and cannulation (see Section **X.2.2.3 Blood Vessel Cannulation**) are the same. Although all casting compounds have unique traits there are general guidelines that should be followed and common pitfalls which should be avoided when casting with any compound.

X.2.1.1 General Guidelines

Before the tissue is removed, a bolus of heparinized saline is injected into the fish to prevent blood clots from forming in the tissue(s) of interest. Heparin concentration varies with species, but for casting the recommended concentration is at least 100 IU/mL. The heparin solution needs to be injected slowly into an anesthetized fish. Once the heparin has been allowed to circulate a couple of times in the fish, the tissue of interest can be cannulated or removed. We recommend cannulating while the tissue is still intact in its original location in the organism if possible and/or submerged in physiological saline as this reduces gravitational distortion of the tissue and inner vascular beds.

The initial cannulation should be done with the largest diameter cannula that will snuggly fit in the desired input vessel. This is because casting material is generally more viscous than blood or saline, and a larger diameter cannula will allow for higher infusion flows at a given applied pressure. If the vessel of interest is small, a large diameter cannula with a

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short tip of smaller diameter is helpful in optimizing tissue perfusion with the casting compound. If the tissue of interest contracts rhythmically, or changes contractile state upon anesthesia, perfusion with a muscle relaxing solution (such as papaverine-containing saline; Hagensen et al. 2008) with heparin prior to casting is highly recommended. This reduces the large variation in quantitative data, such as inter-vascular distances (Cox et al. 2016). For example, when casting the coronary circulation, inter-vascular distances would differ between a cardiac muscle cast in diastole versus systole. Likewise, the contractile state of the vasculature itself can introduce large variation in quantitative data. If quantifying the vasculature is the goal, then flushing the tissue with a vasodilator and monitoring flow rates through the cannula should be considered (McCarter et al. 2015). Flow rates should increase following perfusion with a vasodilator containing saline. Should casting not be possible when tissues are fresh, perfusing the vasculature with 4% buffered paraformaldehyde is recommended. Casting material can then later be introduced to the fixed tissues under a fume hood.

X.2.1.2 Common Pitfalls

The mixing of casting material with saline, fixative, or blood can reduce the structural integrity of the cast and/or dilute the casting material in a way that does not allow for it to be detected by imaging machinery (e.g., micro-CT scanner or fluorescent microscope). This results in "choppy" cast reconstruction by imaging software. To reduce this, a three-way valve can be inserted close to the tissue to reduce mixing in the cannula (one input for saline or fixative, one input for casting material). Air bubbles must be avoided when switching from one solution to the other.

Air bubbles are the enemy of every good cast, and can also cause choppy imaging and/or casts to collapse or flake upon tissue removal (usually accomplished with tissue submersion in 10% KOH). Bubbles are most commonly introduced when mixing casting components. As such, components should be mixed very gently which can sometimes be challenging when cure times are fast. If possible, let the mixed casting material sit for as long as possible before perfusion to let any small bubbles make their way out of the solution. This is because even microbubbles can cause big problems. Small air bubbles can also increase the resistance over capillary beds and once entrenched are difficult or impossible to "push through" the capillaries without damaging the vascular beds. This can lead to venous branches of the circulation to go undetected.

Using supra-physiological pressures to push casting material through the circulation can result in casting material being forced into the intercellular space or cause capillaries or other vessels to rupture. Thus, physiological perfusion pressures should be used when possible. If the viscosity of the casting compound is necessarily very high, moderate hand pressure or an infusion pump can be used. However, the cast must then be carefully checked for indicators of distended vessels or large globular collections of casting material; the latter being an indicator of ruptured vessels or that the casting material has been forced into intercellular spaces. If vessels are found to enter such globular masses and do not exit, then less input pressure is likely required. Alternatively, it could indicate that the vessels drain into a lumen within the tissue (e.g., the coronary circulation draining into the lumen of the heart).

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X.2.1.3 Common Casting Compounds

Mercox is often used for visualization of gross morphology and has a low viscosity allowing for more rapid perfusion. Using a recirculating gentle stream of KOH over and around the tissue aids in the digestion of the tissue without damaging the cast. Mercox casts tend to be quite delicate so extra care must be taken to always keep the tissue submerged once the casting material has cured (2–10 min). Images of the cast can be obtained using a dissection microscope with a camera or via a micro-CT scan following maceration of the tissue (Wirkner and Prendini 2007).

Microfil casts are rubbery and more robust than Mercox with less shrinkage and a longer curing time (20 min), but it is not possible to digest tissue away without harming the material (unless vessel size is over 100 µm and using a special Microfil product). It is possible, however, to make surrounding tissues transparent using methyl salicylate without harming the cast. Microfil was designed to fill microvasculature using physiological pressures and is best used in combination with a micro-CT scanner and imaging software, which allows for the quantification of vascular indexes (McCarter et al. 2015).

VasQtec when cured is flexible and very robust. This material can be easily cut to isolate sections of tissue for analysis (Meyer et al. 2007). It can be used with either a fluorescent microscope for analysis of small 3D vascular sections or with a micro-CT scanner depending on which product is used (Meyer et al. 2007). VasQtec is extremely viscous and moderate hand pressure is often required to perfuse vessels. It has a long cure time (40–60 min), which is needed as bubbles form easily when mixing the components and often take time to leave the solution.

Batson's #17 comes with manufacturer's instructions on how to mix the base, catalyst, and pigment for a recommended viscosity, however, many users modify this recipe depending on the structures of interest (see Aharinejad and Lametschwandtner 1992; Cornillie et al. 2019). This material can withstand the use of 25% KOH (for tissue removal) or 15% sodium hypochlorite (removes tissue but preserves skeletal structures; Cornillie et al. 2019). Scanning electron microscopes and micro-CT scanners have all been used effectively to analyze these casts (Cornillie et al. 2019).

X.2.2 Blood Sampling

Blood can provide a wealth of information about the physiological status of a fish. Researchers can take a blood sample to evaluate osmoregulation (e.g., ions such as Na^+, K^+, see **Chapter 9**), reproductive status (e.g., hormones such as estradiol, testosterone, see **Chapter 12**), epigenetics (e.g., microRNAs such as miR-1, mrR-33a, see **Chapter 3**), endocrinology (e.g., stress hormones such as cortisol, see **Chapter 14**), digestion (e.g., amino acids or fatty acids, see Eliason et al. 2010; Karlsson et al. 2006), immune function (e.g., thrombocytes and granulocytes, see below) and cardiorespiratory function (e.g., **hematocrit** and blood gases, see below).

X.2.2.1 General Considerations when Sampling Blood

There are several considerations to remember when sampling blood from a fish. First, the "ideal" method to obtain blood samples from unaffected and unhandled fish is via cannula-

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tion (see below for details). However, in many cases, cannulation is not possible and single blood sampling is employed. Second, the capture and handling method employed to obtain the single blood sample can profoundly influence blood parameters. For example, a fish that is chased to exhaustion for several minutes, then finally captured in a net to sample blood, will provide very different blood values compared to a fish that is rapidly netted and immediately sampled in less than 30 s. Researchers are encouraged to carefully consider how stress during sampling may influence their blood parameters of interest and to minimize stressful capture and handling, unless the response to stress is the research topic of interest.

Fish may be live-sampled (with or without anesthesia, depending on the situation) or euthanatized prior to blood sampling. A concern for researchers is whether the method of euthanasia may produce artifacts in blood parameters. Chemical methods of euthanasia (e.g., overdose of MS-222) are commonly employed and act by blocking voltage-gated sodium channels, inhibiting nerve conduction and ultimately leading to ventilatory and cardiac arrest. Physical methods of euthanasia are also commonly employed (e.g., blunt trauma to the head with sufficient force to destroy the brain and/or induce loss of consciousness), and are usually considered to have less of an impact on blood parameters. However, the issue is complex as the method of euthanasia has been found to influence some blood parameters in some fishes, but not in others (Holloway et al. 2004; Carter et al. 2011; Weber et al. 2011; Larter and Rees 2017). For example, euthanasia by MS-222 overdose resulted in higher hematocrit compared to other methods (blunt trauma, overdose in clove oil and rapid cooling) in Gulf Killifish *Fundulus grandis* (Larter and Rees 2017). In contrast, blunt trauma resulted in elevated plasma cortisol and glucose levels as compared to chemical anesthesia (clove oil or MS-222) in Rainbow Trout *Oncorhynchus mykiss* (Holloway et al. 2004). One study found that numerous blood parameters collected from the caudal vein of adult Coho Salmon *O. kisutch* were similar when either live-sampled or sampled following euthanasia by blunt trauma (Clark et al. 2011). Therefore, they concluded there was no evidence that tissue trauma from cerebral concussion affected blood values. All told, the method of euthanasia is up to the researcher, local regulations, and will depend on the fish species and experimental question.

Once a blood sample is drawn, ideally, it should be immediately processed for the parameters of interest, keeping in mind that blood may need to be handled differently for some variables (e.g., to measure catecholamines, plasma is diluted in 1 mM of ascorbic acid, flash frozen and stored at –80°C). If processing must be delayed, refrigeration or an ice slurry is preferred to keep the blood at ~4°C (above freezing) until processing can be conducted. However, storage time affects many blood characteristics, even under refrigeration (Korcock et al. 1988; Clark et al. 2011; Faggio et al. 2013). For example, 8.5 h of storage at 0–1°C resulted in an increase in plasma glucose and a decrease in plasma chloride and potassium levels in adult Sockeye Salmon *O. nerka* blood (Clark et al. 2011). As such, researchers should process blood (e.g., centrifuge to separate plasma and erythrocytes) in a standardized way and preferably as quickly as possible after it is drawn. The separated plasma and cell components may be stored at –80°C for several months without affecting ion composition, though enzyme activity decreases with prolonged storage (see Beutler 1975; Beutler et al. 1977 for common red-cell enzyme activity analyses).

Some fish blood coagulates rapidly. To prevent clotting, the cannula or blood sampling syringe is typically flushed with the appropriate fish saline (or simply the appropriate NaCl solution; e.g., 0.9%) that contains an anticoagulant such as heparin or ethylenediamine tetraacetic acid (EDTA). However, blood parameters can be influenced by anticoagulants (Walencik

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and Witeska 2007; Maqbool et al. 2013; Faggio et al. 2014). EDTA has been demonstrated to result in **red blood cell** swelling and hemolysis (Walencik and Witeska 2007; Maqbool et al. 2014). As such, heparin is the preferred anticoagulant for most applications. The concentration varies with species, temperature and procedure but generally ranges between 10 and 200 IU/mL. However, there are notable examples when heparin may be unsuitable. When sampling for microRNAs, EDTA is the preferred anticoagulant since lithium-heparin was found to alter miRNA levels (Glinge et al. 2017). In addition, intravenous injection of heparin releases lipases into the plasma (Skinner and Youssef 1982), rendering heparin inappropriate for some applications.

X.2.2.2 Single Sampling

Once-only live sampling or terminal blood sampling regimes are commonly employed. For large fishes, the most popular approach is a caudal puncture, colloquially referred to as the "grab-and-stab" technique (Figure X.2). This method is used to collect blood from the caudal vessels (caudal artery and caudal vein) located within the hemal arch. Typically, the fish is positioned ventral side up and a needle (usually 21–23 G, with a syringe or Vacutainer) is inserted at an angle along the midline between the anal fin and caudal fin (Figure X.2). If the needle penetrates to the vertebrae, it may be repositioned into the caudal vessels by retracting the needle slightly from the vertebrae. The vessels may also be reached by lateral penetration, from a position slightly ventral to the lateral line. The needle is inserted just anterior to the anal fin and moved in an anterior direction toward the midline. Notably, the caudal blood sample is typically a mixed arteriovenous sample due to the close positioning of the two ves-

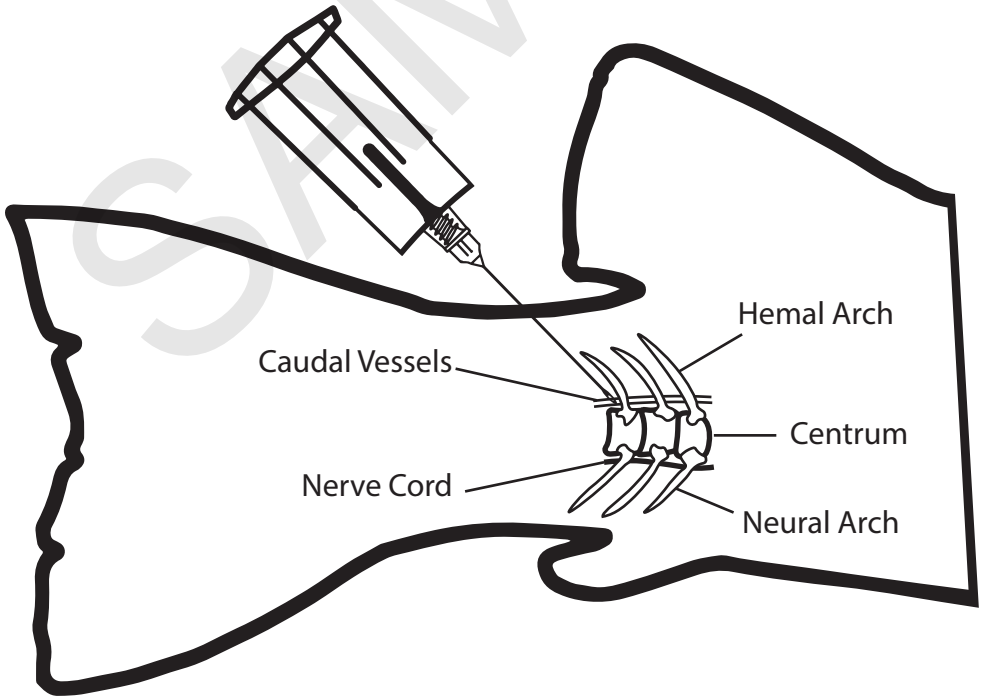

Figure X.2. Blood sampling via caudal puncture. Graphical illustration by Paul Parsons.

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sels. A major advantage of the caudal puncture method is that it can be conducted quickly and without harming the fish, as long as the fish is handled correctly and the appropriate amount of blood is collected (typically less than 10% of the total blood volume). As such, caudal punctures are commonly used to take a single, nonlethal, blood sample from wild fish such as during tagging studies (e.g., Minke-Martin et al. 2018), as well as from fish in laboratory-based and aquaculture studies.

For terminal samples on small fishes, the caudal fin can be severed with a sharp knife or scalpel and then a needle and syringe or microcapillary tube may be used to draw the mixed blood (e.g., Tierney et al. 2004). Direct sampling from the heart, termed a cardiac puncture, is also possible (e.g., Devor et al. 2016), though this method can be ineffective because trabeculae in the heart may block the needle during sampling. If desired, blood can be sampled from any number of vessels (e.g., sinus venosus or dorsal aorta; Tierney et al. 2004) via a single sample using a needle and syringe.

X.2.2.3 Blood Vessel Cannulation

A small tube, or cannula, may be implanted into a blood vessel for repeated blood sampling, blood pressure measurement (see **Section X.2.5**) or to inject pharmaceuticals (see Axelsson and Fritsche 1994 for a detailed description of cannulation procedures). Vessel cannulation methods permit repeated blood sampling from calm, unanaesthetized, free-swimming fish over hours or even weeks, yielding more representative blood samples in unstressed fish. As blood samples are removed, the equivalent volume is typically replaced with the appropriate fish saline. Several different vessels have been cannulated in fish, the major ones are described below.

Before surgery (e.g., to implant a cannula or flowprobe), the fish must first be anesthetized (Neiffer and Stamper 2009), typically via submersion in a bath of buffered tricaine methanesulfonate (MS-222; dose is species-specific) though numerous other anesthetics may be used (e.g., clove oil, benzocaine). During more prolonged surgeries such as vessel cannulation procedures, the gills need to be irrigated with the appropriate maintenance dose of chilled, aerated anesthetic via soft pliable tubing and a water pump in a recirculating bath. The fish must be closely monitored throughout surgery to ensure that the body surfaces do not desiccate and the fish remains in the appropriate plane of anesthesia. The appropriate duration for recovery from surgery will vary with the fish species, surgical procedure, and research question. It may take from minutes, to hours, to several days for different physiological processes to return to baseline preoperative levels (e.g., hematological indices, blood gases, oxygen consumption rate, heart rates, ventilatory rates, ion and osmoregulatory function) (Gräns et al. 2014; Brijs et al. 2018b).

X.2.2.4 Dorsal Aorta

The most common cannulation procedure in fishes is the dorsal aorta cannulation via the mouth. Conte et al. (1963) and Smith and Bell (1964) first described the technique in salmonids using a hyperdermic needle fitted to the tip of the cannula. However, as the sharp needle tip is left inside the vessel this method may cause tissue/vessel damage and is likely more prone to blood clot formation. Thus, a refinement of this technique using an internal

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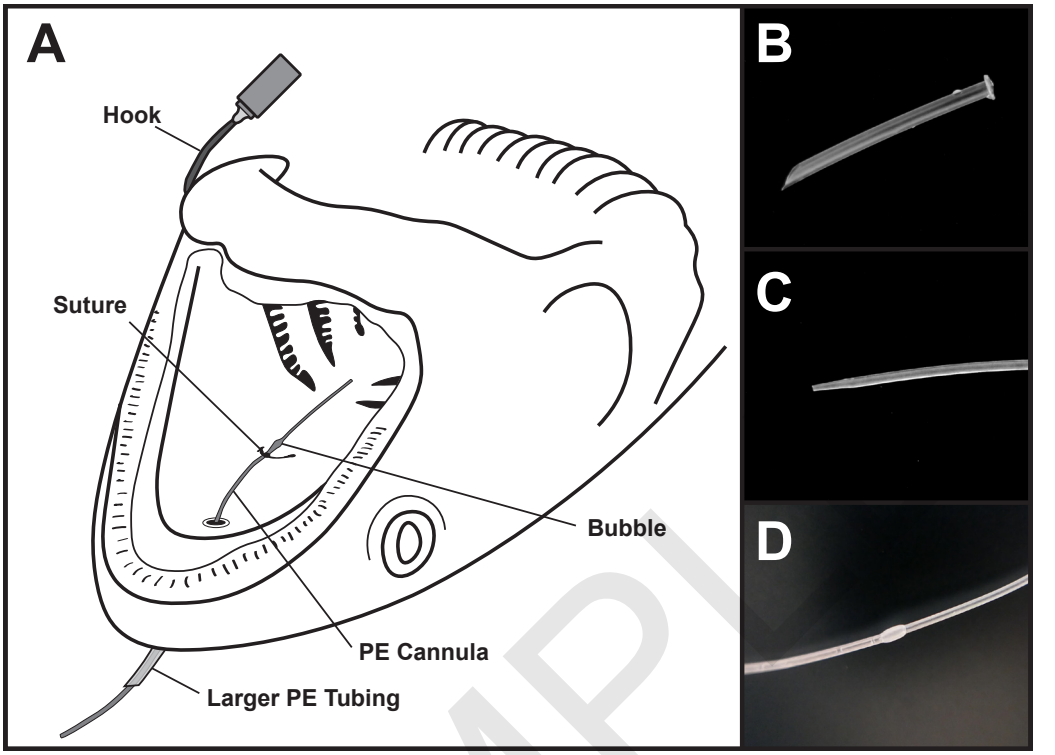

Figure X.3. A) Dorsal aorta cannulation via the mouth. B) Heat flared and beveled PE 190 tubing to exit the cannula via the roof of the head; C) tapered tip of PE 50 cannula and D) bubble in PE 50 cannula. Graphical illustration by Paul Parsons, Photos by Matthew Gilbert.

steel wire as a trochar was introduced by Soivio et al. in 1975 and is still used today (Figure X.3). Polyethylene (PE) tubing is a commonly used material for many cannulation procedures because it is fairly rigid yet flexible, inert, nontoxic and available in a range of sizes. To cannulate the dorsal aorta in salmonids, the PE cannula (of the appropriate size for the blood vessel, e.g., PE-50 in large-bodied fish >500 g) must be prepared by tapering the tip so that it fits tightly around the trochar. This is often done by heating the PE tubing with a flame or soldering iron and stretching the tubing. A small bubble is placed in the cannula wall using a heat source, several cm from the tip (see Figure X.3). The cannula is flushed with heparinized saline solution and then a sharpened stainless steel trochar (e.g., a guitar string, usually 14 or 16 gauge) is inserted into the cannula until ~1 mm of the trochar is protruding from the tip of the cannula. In addition, a short length of larger bore PE tubing (e.g., PE-190) is prepared by heat-flaring one end and beveling the other (Figure X.3).

To begin, a ~12 gauge stainless steel hypodermic needle is driven completely through the roof of the mouth, just anterior to the nostrils, and the larger, flared PE tubing is inserted (Figure X.3). Next, a small hole is made at the site of insertion (using a scalpel or 16G needle), usually just anterior to the first gill arch or between the first and second gill arches (Figure X.3). Then the trochar with cannula is inserted at a ~45° angle, along the midline. The trochar is advanced posteriorly until it penetrates into the aorta (sometimes perceived as a 'pop'). The trochar is then removed, leaving the cannula in place. Correct placement is evident by

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the rapid and continuous appearance of blood in the cannula. The cannula is then advanced several centimeters into the dorsal aorta to ensure it is firmly in place and then flushed with heparinized saline solution. The bubble is anchored to the roof of the mouth via a silk or monofilament suture. The cannula exits the fish's mouth by feeding it through the flared PE tubing in the roof of the mouth.

The tip of the cannula is either sealed by burning the end or plugged with a stainless steel pin. Extensions may be added to the cannula by using broken-off stainless steel needles as connectors and instant glue. The cannula is usually secured along the dorsal surface of the fish with several sutures.

The dorsal aorta may also be cannulated via the efferent branchial artery (e.g., Atlantic Cod *Gadus morhua*, Axelsson and Nilsson 1986; Antarctic fish *Pagothenia borchgrevinki*, Sandblom et al. 2009a) or the tail (e.g., dogfish, Opdyke et al. 1982; Starry Flounder *Platichthys stellatus*, Watters and Smith 1973; hagfish, Axelsson et al. 1990).

X.2.2.5 Ventral Aorta

The ventral aorta may be cannulated using several different methods. A "blind" cannulation can be performed in salmonids by entering via the tongue (Gamperl et al. 1994b). Similar to the dorsal aorta cannulation outlined above, a heat-flared large bore PE tubing is exited through a hole in the side of the mouth. A large gauge needle (e.g., 21 G) is then inserted and rotated into the cartilage of the tongue before the second gill arches. A PE50 cannula with several bubbles and an internal trochar is then inserted into the hole to pierce the ventral aorta. Once blood is freely flowing, the cannula is secured to the fish via the bubbles, as described above.

The ventral aorta may also be accessed via the afferent branchial arteries (Axelsson and Nilsson 1986; Fritsche 1990; Axelsson et al. 1990; and see **Box X.1 Case Study**). To do this, a hole is made (using fine forceps or a needle) between two gill filaments (e.g., the lower area of the 3rd gill arch) and the cannula (cut on a 45° angle for easier insertion, or tightly fitted with an internal trochar) is "blindly" inserted into the afferent branchial artery and advanced toward the ventral aorta. The position is correct if blood is freely flowing. The cannula is then sutured to the gill arch and secured to the body wall.

X.2.2.6 Central Veins

The central venous compartment adjacent to the heart can be accessed by cannulating the Ductus of Cuvier or Sinus venosus. This method has been successfully employed in a range of species including various salmonids (Sandblom et al. 2006b; Clark et al. 2008b; Steinhausen et al. 2008; Eliason et al. 2013b) and European seabass (Sandblom et al. 2005). A PE cannula is prepared by placing several bubbles ~2 cm from the tip, depending on the size of the fish (see above) and the tip is cut on a 45° angle with several side holes in the first cm. The fish is placed on a surgery table ventral side up or on its side. A plastic gill guard and umbilical tape are often used to open the branchial cavity during surgery and to protect the delicate gill tissue from damage. A small incision is made in the tissue between the cleithrum and fourth branchial arch, then the duct of Cuvier is carefully exposed and dissected free. A small portion of the sac-like vessel is lifted using fine curved forceps and grasped with a vascular clamp. The clamp is gently lifted during the cannulation procedure so that a portion of the vessel is

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Box X.1 Case Study

Cardiac oxygen supply has been hypothesized to limit cardiac performance and critical thermal maximum (CT_{max}, a measure of temperature tolerance) in fish during acute warming (Eliason and Anttila 2017). Ekström and colleagues (2016) tested these hypotheses by continuously measuring venous oxygen tension (P_{VO2}, i.e., the luminal cardiac oxygen supply) and cardiovascular variables in vivo during acute warming in European Perch *Perca fluviatilis*. P_{VO2} was recorded using a fibreoptic Firesting optode (Pyroscience, Aachen, Germany) implanted into the ductus of Cuvier (See **Section X.2.2.3** on cannulation), with the probe tip directed towards the sinus venosus. A small incision was made in the isthmus in the opercular cavity to access the ventral aorta. A blood flow probe (Transonic, model 2.5PSL or 2.5PSB, Ithaca, New York, USA) was fitted around the ventral aorta, which allowed for assessments of cardiac output, heart rate and stroke volume (See **Section X.2.6** on blood flow). The fish was also instrumented with a PE31 cannula filled with heparinized saline inserted into the afferent branchial artery of the third gill arch (See **Section X.2.5** on blood pressure). The cannula was forwarded downstream so that the tip of the cannula was positioned close to the bifurcation of the afferent branchial artery from the ventral aorta. This allowed the measurement of ventral aortic blood pressure, which in combination with cardiac output allowed for calculation of cardiac power output and total vascular resistance.

Cardiac output increased with warming due to increased heart rate, while stroke volume was largely unchanged. As expected, ventral aortic blood pressure, cardiac power output and thus cardiac oxygen demand all increased with warming, while the P_{VO2} declined. However, experimentally induced elevation in environmental oxygen availability (i.e., hyperoxia, 200% air saturation) increased the P_{VO2} across all temperatures, which improved cardiac stroke volume and cardiac output, yet, heart rate still declined at similar temperatures as in normoxic perch. Thus, cardiac oxygen supply plays a crucial role in allowing perch to elevate stroke volume at high temperatures, while blood oxygen levels do not appear to limit heart rate at temperatures preceding CT_{max}. The authors concluded that acute thermal tolerance can only partially be attributed to cardiac failure due to oxygen limitations.

(Box continues)

void of blood. Another vascular clamp is secured a few mm from the first. A silk suture is loosely tied around both clamps and then a small nick is made in-between the two clamps. The cannula is inserted and directed anteriorly into the sinus venosus. When using a PE cannula, the first bubble is advanced into the vessel and the silk suture is used to close the vessel around the cannula above the bubble. The cannula is further secured to the body wall via sutures anchored around the remaining bubbles. In addition, in vivo blood oxygen levels can be measured by implanting an oxygen optode in the sinus venosus using a similar approach (e.g. European Perch *Perca fluviatilis* [Ekström et al. 2016a] Rainbow Trout [Farrell and Clutterham 2003] and see **Box X.1 Case Study**). The sinus venosus can also be cannulated "blindly" through the lateral body wall using a PE-50 cannula fitted to an internal trochar (similar to the dorsal aorta cannulation procedure described above), however, great care should be taken

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Box X.1 Continued.

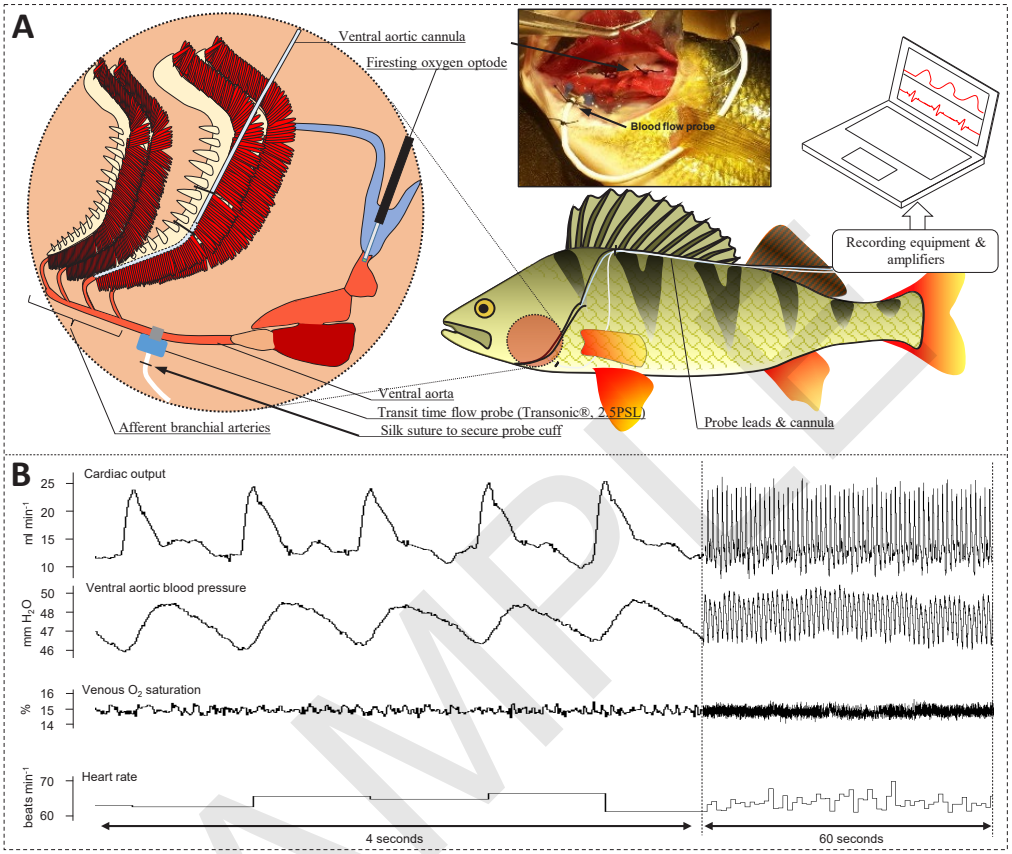

Figure. European Perch *Perca fluviatilis* surgically implanted with instruments allowing for the determination of cardiac output, ventral aortic blood pressure and the partial pressure of venous oxygen during acute warming. A) A European Perch instrumented with a transit-time flow probe around the ventral aorta to record cardiac output, a cannula in the afferent branchial gill artery to measure ventral aortic blood pressure, and a fibre-optic oxygen optode in the ductus of Cuvier to measure the partial pressure of oxygen in the venous blood. The inset photograph depicts a perch instrumented with a ventral aortic cannula and a Transonic blood flow probe. B) Traces (recordings) of resting cardiac output, ventral aortic blood pressure, and O_2 saturation in the sinus venosus of a perch. Heart rate was determined by counting the peaks (i.e., due to ventricular contraction) in the cardiac output trace. Graphical illustration by Andreas Ekström and Albin Gräns. Photo: Erik Sandblom.

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to verify correct placement of the cannula postmortem (e.g. Rainbow Trout [Minerick et al. 2003]; Spiny Dogfish *Squalus acanthias* [Sandblom et al. 2006a, 2009b]; and *P. borchgrevinki* (Sandblom et al. 2008, 2009a).

The hepatic portal vein can be cannulated to sample blood leaving the gastrointestinal tract en route to the liver (Karlsson et al. 2006; Eliason et al. 2007, 2010). The cannula (e.g.,

PE50) is prepared by stretching a short length of silastic tubing (e.g., 0.51 mm ID, 2–3 cm long, depending on the size of the fish) over the end of PE tubing that contains several bubbles (~2 mm, 5 cm, and 7 cm from the end). Small holes are made in the sides of the silastic tubing and the tip is cut on a 45° angle to ease insertion. The fish is placed ventral side up on the surgery table and a scalpel blade and Mayo scissors are used to make an incision posterior to the pectoral fin between the ventral midline and lateral line. The incision site is held open with retractors. A side branch of the ventral or dorsal intestinal vein is identified for cannulation and an ~0.75 cm portion is isolated free using sterile swabs and fine curved forceps. Two pieces of silk are placed around the vessel; the posterior piece is secured tightly to occlude the vessel while the anterior piece is tied loosely. A small nick is made in the vessel, taking care not to transect the vessel. The silastic tip of the cannula is directed into the vessel and advanced into the hepatic portal vein (i.e., toward the liver). The two pieces of silk are tightened around the first bubble to secure the cannula. The incision is closed (e.g., using interrupted monofilament sutures) and the cannula is secured to the body wall of the fish via the remaining bubbles (for the detailed procedure, see Eliason et al. 2007).

X.2.3 Routine Hematological Methods

Blood composition is routinely measured to evaluate a fish's physiological status (e.g., response to exercise or an environmental change) or as a diagnostic tool to assess fish health (e.g., anemia, Blaxhall and Daisley 1973). The main role of red blood cells' (RBCs) is to transport oxygen to the respiring tissues. **White blood cells** (WBCs) are primarily involved in immunity and immunological defenses.

X.2.3.1 Hematocrit & Leucocrit

To determine the fraction of blood that is composed of RBCs (hematocrit) or WBCs (**leucocrit**), blood is drawn via capillary action into a microhematocrit tube and sealed with commercial sealant (e.g., Critoseal). The tube is placed in a hematocrit centrifuge for 5 min, typically at 7,000–10,000g. Hematocrit can then be determined using a microhematocrit reader or a standard ruler using the following calculations: hematocrit = [packed red cell layer/(packed red cell layer + packed white cell layer + plasma layer)] ×100; leucocrit = [packed white cell layer/(packed red cell layer + packed white cell layer + plasma layer)] × 100. Across fishes, hematocrit typically varies between 10 and 40% (though it is 0% in icefishes) and leucocrit typically varies between 0.3 and 1.0% (Farrell 2011). Notably, hematocrit is an unreliable estimate of hemoglobin concentration in fishes because hemoglobin concentrations vary considerably across fishes and fish RBCs can swell with stress (thus, hematocrit may increase with no increase in hemoglobin concentration).

X.2.3.2 Hemoglobin

Blood hemoglobin concentration is traditionally measured by the cyan-metahemoglobin method, commonly referred to as the Drabkin method (Drabkin and Austin 1935). Blood (typically 10–20 μL) is added to a small aliquot of Drabkin's reagent (e.g., 1–2.5 mL) and mixed with a vortex. The cyanide derivative in the Drabkin's solution hemolyses the red

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blood cells and the hemoglobin derivatives are oxidized by ferricyanide to produce methemoglobin, which forms a stable complex with cyanide. The mixture is centrifuged for 3–10 min at 3,000–10,000g to separate out degraded proteins. The absorbance of the stable cyanmethemoglobin complex is measured on a spectrophotometer at 540 nm. One drawback of this method is that it is laborious and sometimes ill-suited for field conditions. In response, a handheld hemoglobin analyzer (HemoCue) developed for human blood was validated for fish blood against the Drabkin method (Clark et al. 2008a). A blood sample is drawn into the HemoCue cuvette by capillary action and spontaneously mixes with the reagents in the cuvette. After a standardized duration of time (between 1 and 10 min), the cuvette is placed in the handheld meter. The value indicated on the meter overestimates hemoglobin by 21–24% and must be calibrated for fish (see Clark et al. 2008a). If a researcher has measured both hematocrit and hemoglobin concentration, the **mean corpuscular hemoglobin concentration** (MCHC) can be calculated. This represents the concentration of hemoglobin within an RBC, commonly measured in g/dl: MCHC = [whole blood hemoglobin]/(hematocrit).

X.2.3.3 Leukocytes

While leucocrit provides a coarse and easy measure of WBC abundance, researchers often desire a more detailed analysis of the four major types of WBCs: lymphocytes, thrombocytes, granulocytes, and monocytes. The differential white blood cell (DWBC) count technique is relatively simple to perform (Ellis 1977; Tierney et al. 2004). A drop of blood is placed on a microscope slide, the blood is smeared across the slide and allowed to air-dry. The slide is stained (e.g., Wright-Giemsa or Jenner-Giemsa stain) and leukocytes are counted using a microscope. The major limitation of this technique is that it can be difficult to classify certain leukocytes (e.g., small lymphocytes and thrombocytes can be indistinguishable) and leukocytes vary both inter- and intra-specifically, thus it has been challenging to develop standards (Tierney et al. 2004).

X.2.3.4 A Cautionary Note on Point-of-care Devices

Portable medical analytical instruments have become increasingly popular among fish researchers to assess blood parameters (Stoot et al. 2014). Originally developed for medical diagnostic purposes with humans, these so called ‘point-of-care’ devices are popular with fish biologists because they can be used in remote field settings with minimal training and can yield numerous blood parameters from a single blood sample (e.g., blood pH, hematocrit, [hemoglobin], blood gases, $HCO3^{-}$, [Na^{+}]). However, researchers must exercise great caution because the devices were developed for human blood, and thus, often return incorrect values because of fundamental differences in fish and mammalian blood including temperature, nucleated RBCs in fishes versus nonnucleated RBCs in humans, different pH effects on hemoglobin in teleost fishes (i.e., Root and Bohr shifts), and the detection range is optimized for human blood parameters (see (Harter et al. 2014). The most popular device is the i-STAT (Abbot Point of Care Inc., Princeton, NJ, USA); over 30 studies have used the i-STAT system with elasmobranchs or teleosts and it is commonly used for other taxa as well (Harter et al. 2014; Stoot et al. 2014). Even still, the i-STAT system was found to yield inaccurate results for most variables in Rainbow Trout (hematocrit, Na^{+}, PCO_2, $HCO3^{-}$, PO_2) and for blood

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gases in sandbar sharks (PO_2 and PCO_2) (Harter et al. 2014, 2015), and thus, is not recommended for these variables. It did, however, provide accurate results in both species for blood pH after a conversion factor was applied (Harter et al. 2014; Harter et al. 2015). Other single variable point-of-care devices are also commonly used (see Stoot et al. 2014 for a review). For example, point-of-care devices for blood pH (Hanna Instruments pH meter HI-99161, Woonsocket, RI, USA), lactate (Lactate Pro LT-1710 Akray Inc, Kyoto, Japan), glucose (Accu-Check Compact Plus; Roche Diagnostics, Basel, Switzerland) and hemoglobin concentration (see above) have been validated for fishes and elasmobranchs (Wells and Pankhurst 1999; Awruch et al. 2011; Stoot et al. 2014). In summary, point-of-care devices can be useful tools for fish biologists as long as they are accurately calibrated for use with fish blood.

X.2.4 Heart rate

The rate at which the heart contracts is a central cardiorespiratory variable that is affected by most behavioral responses and environmental perturbations (Costa and Sinervo 2004; Cooke et al. 2016). Because of the sensitivity of heart rate, and its fundamental role in circulatory oxygen convection, it has been widely used as an indicator to quantify and assess e.g., activity, metabolic rate, food intake, digestion, predator presence, thermal tolerance, and as a general stress indicator in fish (Armstrong 1986, 1998; Höjesjö et al. 1999; Altimiras and Larsen 2000; Clark et al. 2010; Gräns et al. 2014; Anttila et al. 2014). There are several methods available to measure heart rate in fish. However, the research question, and technical limitations, often dictate the selection of the recording technique. Below is a summary of the most commonly used methods to assess heart rate in fish. In addition to the methods listed below, heart rate can also be determined from measurements of both pulsatile blood pressure and blood flow, which are described later in this chapter and elsewhere (see Sections **X.2.5 and X.2.6**; Stevens and Randall 1967; Eliason et al. 2008; Gräns et al. 2009a).

X.2.4.1 Visual Determination of Heart Rate

Heart rate can be determined visually, either directly or from video recordings, in small species, as well as in larvae and embryos, which are often translucent (Barrionuevo and Burggren 1999; Ishimatsu et al. 2004). This method gives a relatively rough measure of heart rate but can be very useful when looking for quite large effects or even as an indicator of death (i.e., the cessation of heart beat; Ishimatsu et al. 2004).

X.2.4.2 Electrocardiogram (ECG)

A more detailed and perhaps informative method to measure heart function and rate is through measurements of the electrical signals associated with the cellular repolarization/depolarization events. This is called an **electrocardiogram** (ECG) and has been measured in a range of different species of fish (Oets 1950; Roberts et al. 1973; Nomura and Akiyama 1984). In general, the ECG of fish looks largely like that of other vertebrates with distinct P, Q, R, S, and T peaks (see Figure X.4). The P peak represents atrial depolarization, which initiates atrial contraction. The QRS complex indicates the depolarization and contraction of

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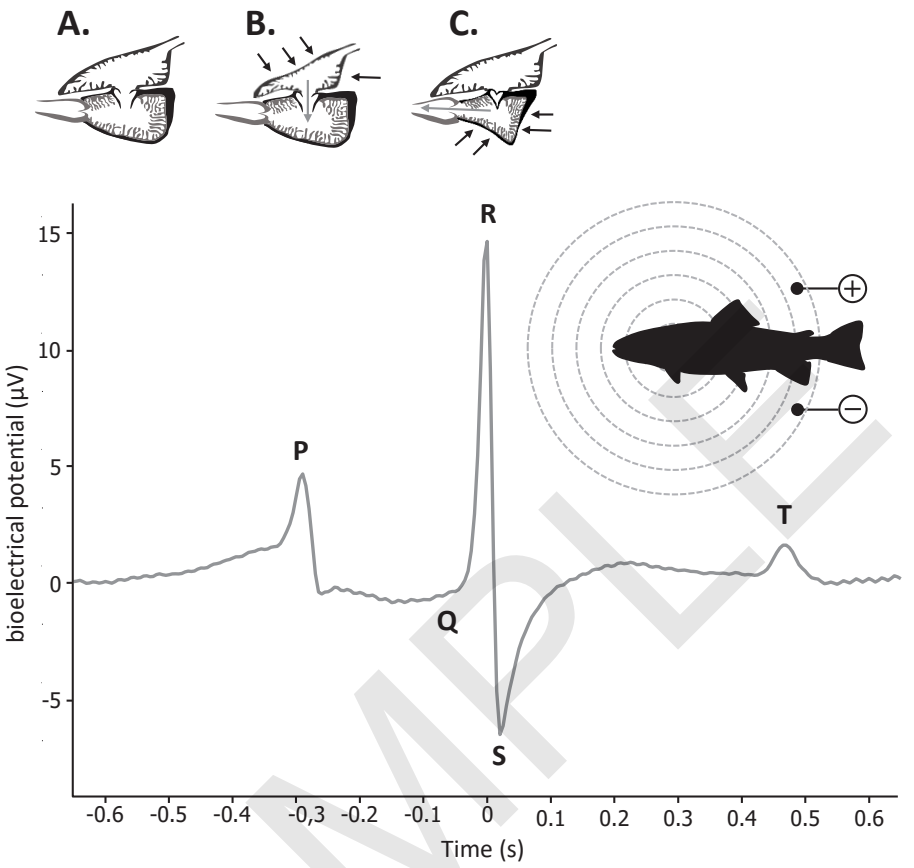

Figure X.4. The electrocardiogram (ECG) of a Rainbow Trout *Oncorhynchus mykiss* measured in the surrounding water of a free-swimming fish with the use of external electrodes (indicated by the + and –). The trace is an average of 120 heart beats and shows distinct peaks for P, Q, R, S, and T. The relaxed heart during repolarization is shown in A. The atrial contraction (the P peak) is shown in B and ventricular contraction (the QRS complex) during ventricular systole is shown in C. Black arrows symbolize the contraction of the heart chambers and gray arrows symbolizes the direction of blood flow due to intra-cardiac pressure differences.

the ventricle and normally begins with a small negative deflection, the Q peak, followed by a larger upwards deflection (the R peak) and ends with the downward S peak (see Figure X.4). The final part of the ECG is a smaller upwards waveform (the T peak), which represents ventricular repolarization. The P-Q interval indicates the period between the atrial and ventricular contractions. The length of the Q-T interval represents the overall duration of ventricular activity. The amplitude of the QRS complex reflects the force of ventricular contraction, but it is also largely affected by the recording method and equipment used, as well as by subsequent signal filtration. Thus, quantitative comparisons of signal heights both between and within studies can be problematic. There is also a ST segment that connects the QRS complex and the T peak that represents the isoelectric period between depolarization and repolarization of the ventricle.

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The ECG can be used to determine heart rate (typically calculated from the R-R intervals), and to acquire information regarding the heart's condition such as arrhythmic heart beats, heart attacks, pacemaker function, and heart failure. For example, an elevated ST segment is a sign of heart failure caused by myocardial ischemia, as observed during coronary obstruction in Rainbow Trout (Ekström et al. 2017). For a comprehensive guideline on the diagnosis of acute and chronic heart failure in human patients, see Ponikowski et al. 2016. While these guidelines are developed for humans, they are also largely applicable to other vertebrates. Longer recordings (preferably hours) of ECG can also be used to determine the regularity or variability of the R-R intervals. This is normally referred to as the heart rate variability (HRV), and is influenced by the activity of the autonomic nervous system (Altimiras 1999; Gräns et al. 2014). Heart rate variability is commonly used to determine the level of recovery after e.g., exercise, trauma and stress in mammalian studies, but practical applications of this analysis in fish are still sparse (but see (Campbell et al. 2004; Jeanne et al. 2009; Gräns et al. 2014). With the recent intensification of Zebrafish *Danio rerio* as a model organism to study human disease and medicine, examination and recording of ECG in this species has become an area of research with great potential (Liu et al. 2016).

X.2.4.3 Methods for Measuring ECG

There are several different techniques that can be used to measure ECGs in fish. The general equipment needed are electrodes, a bio amplifier and a data acquisition device. One lead (i.e., the voltage difference between two electrodes) is most commonly used to measure the ECG in fishes. Additional leads can be used to improve ECG definition, but are seldom used in studies on fish. The material used for electrodes should exhibit high conductivity. Other important properties to take into consideration when selecting electrode material are corrosion resistance and toxicity. These are particularly important when the material is operating in exposed environments such as in seawater and therefore, stainless steel electrodes are most commonly used today.

There are no general guidelines for how to position the electrodes that applies for all fish species, and different researchers often have their own techniques, which are all slight modifications of the methods listed here. One common position is to implant electrodes subcutaneously in the ventral surface above the heart with one of the electrodes oriented towards the head (cranially) of the fish and the second electrode oriented towards the tail (caudally); i.e., with the heart located in a cranially-caudally position between the electrodes (see Figure X.5). This method works well in Rainbow Trout (Ekström et al. 2016b), Northern Pike *Esox Lucius* (Armstrong et al. 1989), European Perch (Sandblom et al. 2016a) and Zebrafish (Liu et al. 2016). Another common positioning of the lead is with the electrodes inserted percutaneously between the pectoral bones, one on each side of the heart so that the electrode tips lie immediately adjacent to the pericardium with the heart located in a lateral position between the lead (see Figure X.5). This method works well in African Lungfish *Protopterus annectens* (Sandblom et al. 2010) and Leopard Shark *Triakis semifasciata* (Lai et al. 1990). If the fish remains anesthetized during the experiment, the fish may simply be placed ventral side down, resting directly on the two electrodes without any need for sub-cutaneous implantation (Anttila et al. 2014). Some species, such as cartilaginous fishes and lungfishes, are notoriously more difficult to obtain good ECG signals. In these species, positioning of the electrodes very close to the pericardium is recommended (Sandblom et al. 2010).

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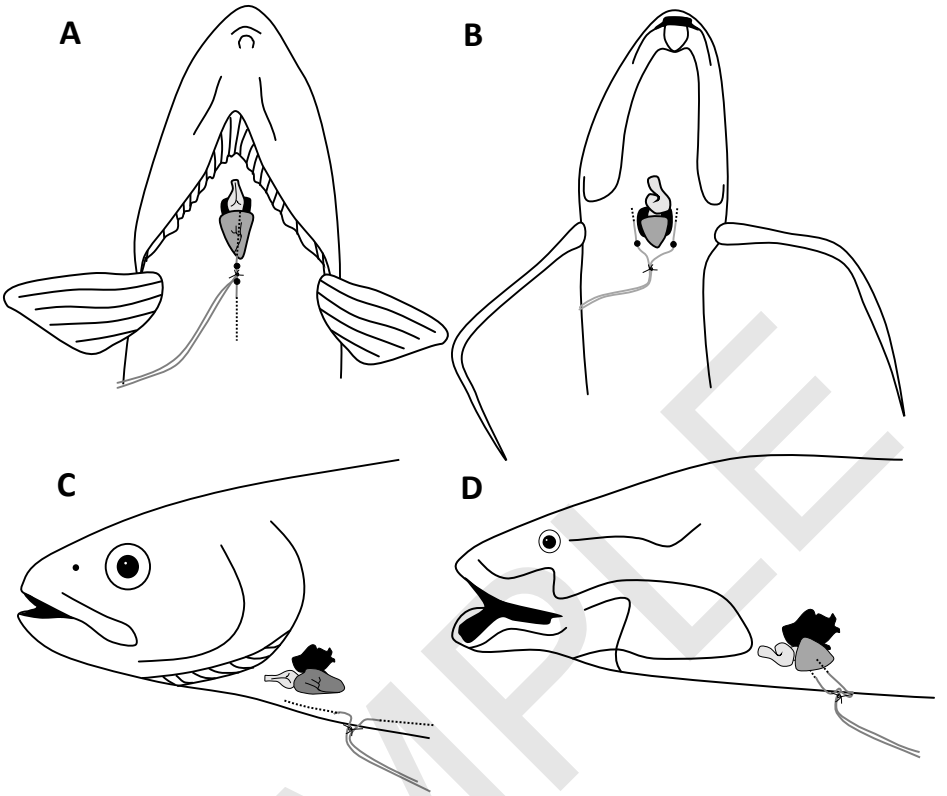

Figure X.5. A and C show the two subcutaneously implanted ECG electrodes in a cranial-caudal position with respect to the heart in a Rainbow Trout *Oncorhynchus mykiss*. B and D show the electrodes inserted subcutaneously so that the electrode tips lie immediately adjacent to the pericardium in a lateral position to the heart in an African Lungfish *Protopterus annectens*. The black circles in A and B indicate the electrode entry holes.

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X.2.4.4 Heart Rate and ECG Recordings in Free-swimming Fish with Bio-logging and Wireless Techniques

Records of heart rate can be obtained either from tethered fish as explained above, or from free-swimming fish using various bio-telemetry or bio-logging devices. The ECG can also be recorded with electrodes positioned in the water adjacent to the fish. Most commonly used in free-swimming fish are heart rate sensors that detect the ECG (Roberts et al. 1973; Priede 1983; Armstrong et al. 1989), but there are also examples of implants that detect heart rate from blood flow measurements (Gräns et al. 2009b, 2010) or from the physical movement of the beating heart (Clark et al. 2008c). However, when inserting the implants, care must be taken not to put excessive pressure against the pericardium, as this may directly affect the function of the heart and potentially cause pericardium lesions that would severely impair heart function (Farrell et al. 1988). Most devices have been developed for specific studies within specific research groups, but there are a few devices that are commercially available. Exceptions to this are the commercially available heart rate storage tags from Star Oddi, acoustic telemetry devices by Thelma BioTel and Vemco, and a radio telemetry system from Transonic.

Some devices detect and record or transmit the entire ECG waveform, while others process the ECG into a mean heart rate over defined durations, which is then stored for later download and analysis (Prystay et al. 2017; Brijs et al. 2018b; Ekström et al. 2018). The advantage of saving heart rate readings instead of the actual ECG recordings is that it dramatically reduces the required data storage space and/or the amount of data that needs to be transmitted. The major drawback is that it omits both the actual raw data and information regarding heart rate variability, which prevents the diagnosis of e.g., acute and chronic heart failure.

Implanting electrodes or other devices requires a surgical procedure. In freshwater species, however, this can be circumvented by using noninvasive methods for cardioventilatory recordings where the weak bio-potentials produced by the heart and ventilatory muscles are recorded directly from the water using paired external electrodes (Goodman and Weinberger 1971). With a simple stainless steel grid placed on the bottom of the tank acting as one of the electrodes, and stainless steel wires placed immediately below the water surface acting as the second electrode, the fish can move freely between the two electrodes without noticeable effects on the quality of the signal (Gräns et al. 2014). When using this method, the ECG needs to be filtered out from the normally much slower rhythm generated by the ventilatory musculature (see Figure X.4) (Altimiras and Larsen 2000; Casselman et al. 2012; Gräns et al. 2014). This can be achieved with the use of an appropriate high-pass filter, e.g., in Rainbow Trout at 10°C, a cut-off frequency of 10 Hz is often appropriate (Gräns et al. 2014).

X.2.5 Blood Pressure

The heart generates the blood pressure that drives the flow of blood to the tissues. Blood pressure, in turn, inherently depends on the resistance of the circulation, which is controlled by neural and endocrine mechanisms, as well as local factors in the tissues (Sandblom and Gräns 2017). The recording of blood pressure can yield important information about both cardiac and vascular function in fish. For example, simultaneous recordings of ventral aortic blood flow (i.e., **cardiac output**, CO) and blood pressure can be used to assess cardiac workload (i.e., cardiac power output, for details, see Farrell et al. 1996; Cox 2010; Ekström et al. 2016a; Sandblom et al. 2016a; Cox et al. 2017; a proxy for cardiac oxygen demand (Driedzic et al. 1983). While recordings of CO and dorsal aortic blood pressure allow for quantification of systemic vascular resistance and overall perfusion (Sandblom and Axelsson 2006; Sundell et al. 2018), determinations of blood flow and blood pressure in tissue specific vessels (e.g., arteries supplying the gastrointestinal tract) yield information regarding local tissue perfusion and resistance. Overall branchial vascular resistance can be quantified by dividing the difference between ventral aortic and dorsal aortic blood pressure by cardiac output (Axelsson et al. 1994; Olson et al. 1997).

Blood pressure in fish is typically recorded by cannulating the vessel of interest with an ample-sized cannula filled with heparinized saline (to prevent blood clotting issues, see **Section X.2.2.1**), which connects and relays the pressure to a pressure transducer (manometer) (Figure X.6). Many blood pressure transducers are of the strain-gauge type sensor, where the electrical resistance varies with applied force (e.g., pressure changes) and produces an electrical signal that can be relayed to a recording device via an amplifier. To obtain reliable recordings of the blood pressure dynamics it is important that the frequency characteristics (dP/dt) of the cannula-manometer system, which is mainly governed by the resistance and capacitance of the system, exceed that of the model system of interest. Cannula characteristics may play an important role

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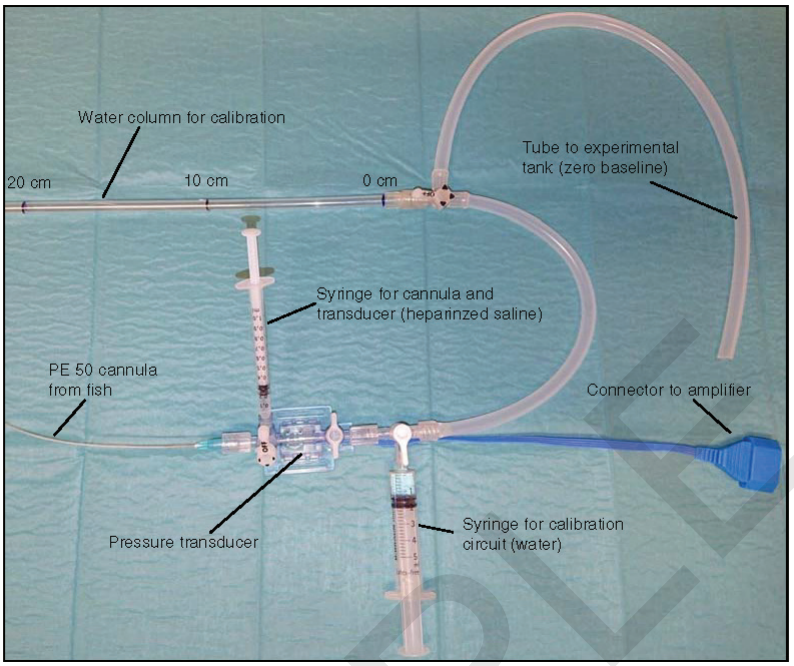

Figure X.6. Image of equipment used to perform blood pressure measurements, including a water column for calibration, a pressure transducer and associated syringes, and the amplifier connection.

in governing the frequency response, as cannula length positively correlates with resistance, while an inverse relationship is true in terms of the inner diameter of the cannula. Thus, the frequency response decreases with decreasing inner diameter and length of the cannula, and thus, using appropriately sized cannulas for specific model systems are of crucial importance (see Axelsson and Fritsche 1994). Thus, the use of custom-made cannulas may need to be considered when assessing pressure in smaller vessels or specimens, to circumvent or minimize potential frequency issues. For example, a short length of smaller diameter cannula fitting into a vessel of interest, can be joined with a larger diameter cannula to reach the recording system, such that overall cannula resistance is reduced. PE cannula material of different dimensions can be joined by gluing, e.g., using Loctite cyanoacrylate (403) and primer (Loctite 770). Moreover, PE cannulas can be modified by pulling and bending the tip of the cannula into a smaller diameter and to better fit the curvature of the vessel of interest. This is facilitated by heating the PE material beforehand. However, such procedures may come at the expense of losing information regarding the dimensional characteristics of the cannula. The stiffness of the cannula material should also be considered, as more elastic materials may dampen the frequency response, especially at high temperatures where the cannula material may become more flexible. Moreover, the frequency response is affected by obstructions affecting the membrane displacement in the recording system. For example, air bubbles trapped in the cannula or in proximity to the pressure transducer exhibit high compressibility that will greatly dampen the pressure changes recorded by the system (Figure X.6). This may not be so much of an issue if only mean pressures are of interest, but if accurate recordings of pulse pressure (i.e., the difference between systolic and diastolic pressures) are required these aspects require careful consideration.

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A liquid filled cannula-pressure transducer system applied in fish studies is typically two-point calibrated against a static water column connected to the transducer via a three-way valve (Figure X.6). The upper point should be set to exceed the maximum pressure range of the experimental model system, using a water column of adequate height. The zero pressure calibration point is the pressure at the surface of the water in the tank containing the fish. Thus, the blood pressure is sometimes reported in units of cm H_2O (1 cm H_2O = 0.7356 mm Hg = 0.1333 kPa). This also means that any change in the water surface of the fish tank, or a change in position of the pressure transducer relative to the water surface, will greatly affect the blood pressure recording, unless the system is re-calibrated at the new position. Indeed, a very stable water surface is required for reliable blood pressure recording. These considerations are particularly important in fish species with low arterial pressures (e.g., hagfish and some inactive benthic teleost species (Axelsson et al. 1990; Cox 2010), and especially when recording venous blood pressures in fish (Sandblom and Axelsson 2007).

X.2.6 Blood flow

X.2.6.1 The Fick Method—a Theoretical Approach of Assessing Blood Flow in Fish

The Fick principle (Adolph Eugene Fick, 1829–1901) can be applied to estimate blood flow to systemic tissues (i.e., cardiac output, CO). Applied in a whole animal cardiorespiratory context, the Fick principle states that CO equals the quotient of the **whole animal tissue oxygen consumption** (MO_2), divided by the arterio-venous oxygen content difference, which is the difference in oxygen content of the arterial blood entering the tissues (C_{aO2}) and of the venous blood leaving the tissues (C_{vO2}). Thus, simultaneous recordings of these metrics can be used to estimate CO in fish [$CO = MO_2/(C_{aO2}–C_{vO2})$ e.g., see **Chapter X Respirometry** and **Section X.2.2.3** above]. However, great caution must be used when considering this approach as not all oxygen extracted from the water by the fish enters the arterial circulation, which may lead to overestimations of CO. First, the extent to which fish rely on cutaneous respiration (i.e., extract oxygen from the environment via the skin) varies considerably within species, between species, and over the lifecycle (Glover et al. 2013). Secondly, some oxygen is consumed directly by the gill tissue, and thus, do not contribute to C_{aO2}. In addition, both cutaneous oxygen uptake and direct branchial respiration may vary across activities and environmental temperatures (Farrell et al. 2014). The fact that some proportion of CO may bypass sections of the lamellar surface areas of the gills participating in oxygen uptake (Hughes et al. 1982; Ishimatsu et al. 1988; Sundin and Nilsson 1992; Olson 2002), could therefore instead result in underestimating CO with the Fick equation. Thus, a full estimation of CO requires additional assessments of branchial-cutaneous oxygen uptake included into a modified Fick equation, which takes these factors into account (Farrell et al. 2014).

X.2.6.2 Direct Approaches for Determining Blood Flow in Fish

There are several direct approaches, both surgically invasive and noninvasive, to quantify blood flow in fish. Here, the principles of the most common techniques are covered. For descriptions of older methods less commonly used in fish research in recent decades, e.g., the dye-dilution and thermal equilibrium methods, we refer to the previous edition of this book (Houston 1990).

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X.2.6.3 Magnetic Resonance Imaging

Nuclear magnetic resonance (NMR) imaging represents a surgically noninvasive method for assessments of relative blood flow in fish. This technique uses spectroscopic visualization of atomic nuclei in tissue, which when subjected to a magnetic field, causes shifts in energy states and in the relative absorption and emission of radio waves, which are detected by radio-frequency transceivers. The fish is placed in a water reservoir positioned inside a magnet bore, and changes in flow can be determined by quantification of the relative changes in signal output over time from the tissue of interest, e.g., a blood vessel. Thus, NMR allows for blood flow determinations without the need for surgical intervention, and can be used to assess flow in small, and by other methodologies, inaccessible vessels. However, the method is highly sensitive to movement artifacts, and thus, requires immobilization of the test subject during the procedure. This is achieved by either anesthesia or physical and/or confinement restraints, which may potentially bias the reliability of the acquired data due to stress associated with such procedures. Moreover, the use of NMR in fish studies is further complicated by the fact that the technique is sensitive to disturbances caused by water flow around the area of focus, as well as by water ionic content and conductance (Van der Linden et al. 2004). Three studies reported relative changes in dorsal aortic blood flow in response to acute warming for nonanesthetized restrained fish using NMR imaging, including Atlantic Cod (Lannig et al. 2004), eelpout *Zoarces viviparus* (Pörtner and Knust 2007) and Antarctic eelpout *Pachycara brachycephalum* (Mark et al. 2002). However, both Mark et al. (2002) and Lannig et al. (2004) reported that cardiac output does not increase with warming beyond 7°C in eelpout and cod. These findings contrast previous studies using more direct recording techniques demonstrating that cardiac output generally increases with warming in fish (see Eliason and Anttila 2017). These contradicting results thus question the reliability of this method for use in fish, especially when tested at different temperatures.

X.2.6.4 Epi-illumination Microscopy

Epi-illumination microscopy represents a technique suitable to assess microcirculatory blood flow in anesthetized fish. The technique involves microscopically visualizing vasculature by illuminating it from above. When applied to fish, a water submersible microscope objective capable of sufficient magnification for identification of individual erythrocytes is required. Blood flow velocity is assessed by quantifying the time required for individual erythrocytes to travel a certain distance. The method has been used to quantify arterial filamental and lamellar blood flow in the gills of Rainbow Trout (Sundin et al. 1995; Sundin and Nilsson 1998) and Epaulette Sharks *Hemiscyllium ocellatum* (Stensløkken et al. 2004), as well as cerebral blood flow in Crucian Carp *Carassius carassius* (Nilsson et al. 1994), Rainbow Trout (Söderström and Nilsson 2000; Haraldsen et al. 2002) and Epaulette Sharks (Söderström et al. 1999). Moreover, Pelster and colleagues used a similar but slightly modified technique to assess blood flow and erythrocyte distribution to somatic tissues in Zebrafish larvae (Schwerte and Pelster 2000; Schwerte et al. 2003; Egg et al. 2014).

Besides potential confounding effects of anesthetics, which may affect cardiovascular function, application of this method may also require more or less invasive surgical interventions when accessing the vasculature of interest.

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X.2.6.5 Blood Flow Probes

By far the most common technique to determine blood flow in fish in recent decades involves the use of flow sensors integrated into cuffs (i.e., flow probes) that are mounted around the blood vessel of interest. Thus, this technique requires invasive surgical interventions, which requires additional precautions, e.g., that the cuff is biologically inert, and for long-term implantation, aseptic and sterile surgery should be considered. Care must also be taken not to damage surrounding tissues when preparing a vessel for instrumentation. For example, damaging nerves may result in confounding effects on subsequent blood flow recordings, and breaching of the pericardium when dissecting the ventral aorta impairs cardiac function(s) in fish (Farrell et al. 1988; Franklin and Davie 1993), especially in elasmobranchs that have a particularly rigid pericardium (Sandblom et al. 2006a). Moreover, it is crucial to consider whether a species´ vascular anatomy will allow for accurate blood flow measurements. For example, to accurately determine total ventral aortic blood flow (i.e., CO), it is important to position the flow probe downstream of the point where the ventral aorta exits the pericardium, and upstream of the first bifurcation of afferent branchial arteries. In some species of sharks, however, the afferent branchial arteries diverge from the ventral aorta inside the pericardial cavity (De Iuliis and Pulerà 2011), and thus, determination of the entire cardiac output using flowmeters is impossible without breaching the pericardium.

The first in vivo blood flow recording in intact fish using implantable flow probes was made in 1962 by Kjell Johansen and coworkers, who used an electromagnetic flowmeter to assess beat-to-beat ventral aortic blood flow (i.e., CO) in Atlantic Cod (Johansen 1962). This technique was pioneered for blood flow measurements by Alexander Kolin, and uses Faraday's law of induction to assess absolute vascular flow rate by quantifying the electromotive force detected by electrodes integrated into a vascular cuff (Kolin 1959). The electromotive force is created when the conductive constituents of the blood pass through an electromagnetic field, produced by an electromagnetic coil. Since Johansen´s experiments, electromagnetic flowmeters have been used to assess blood flow dynamics in numerous teleost and elasmobranch species. This includes determinations of CO via the ventral aorta or afferent branchial arteries (Satchell and Johnes 1967; Satchell et al. 1970; Hemmingsen and Douglas 1972; Axelsson et al. 1989b), gastrointestinal perfusion by the celiacomesentericartery (Axelsson et al. 1989b) and pulmonary artery blood flow in African lungfish (Axelsson et al. 1989a). However, the use of this technique has now been largely replaced by ultrasonic flowmeters, in part due to the difficulties with achieving zero-point calibration values when used for in vivo applications. Moreover, absolute flow determinations with electromagnetic flow probes require complicated and laborious in situ calibration of the probe to the relevant flow range after the experiment.

By far, ultrasonic flowmeter techniques have been the most commonly used method for assessing blood flow in fish in recent decades. The pulsed Doppler technique allows measurement of relative changes in blood flow velocity by assessing the change in frequency of the echo of a pulse of ultrasonic sound emitted from a transducer (a small piezoelectric crystal), that is transmitted through the blood vessel at a ~45° angle. The transducer also acts as a receiver for the sound´s echo, which undergoes a change in frequency proportional to the flow velocity of circulatory erythrocytes (i.e., Doppler shift). The main advantage of Doppler probes is that they are relatively inexpensive and custom-made cuffs integrating the transducer can be

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designed for a precise and snug fit on both large and small vessels of interest. Moreover, the length of the probe lead can be adjusted in length to suit the intended application, and the probe leads are lighter and more flexible compared to, for example, Transonic flow probe leads. However, similar to electromagnetic flow probes discussed above, acquiring absolute flow values using the Doppler technique requires in situ calibration after the experiment (Axelsson et al. 1992; Clark et al. 2007). To circumvent this cumbersome step, many studies simply report relative changes in blood flow, which in many cases is sufficient to address the research objectives. Over recent decades, the Doppler technique represents one of the more prevalent methods for assessing absolute and relative blood flow in large and small vessels in fish including the ventral aorta (Axelsson et al. 1992; Gamperl et al. 1994b; Sandblom et al. 2006b; Clark et al. 2007; Ekström et al. 2014), the coronary artery (Farrell 1987; Axelsson and Farrell 1993; Gamperl et al. 1994a, 1995), the celiacomesenteric artery (gastrointestinal tissues; Holmgren et al. 1992; Thorarensen et al. 1993; Brijs et al. 2015), the hepatic portal vein (hepatic tissues; McLean and Ash 1989), and the pseudobranchial artery (Waser and Heisler 2004).

Transit-time flowmeters also emit an ultrasound signal through the vessel via a transducer (piezoelectric crystal), but the signal is reflected back to a receiver via a reflector. This technology allows for determination of the volume flow per unit of time, by assessing the phase shift in transit-time (acceleration/deceleration) of the signal when passing through the vessel and its constituents (blood plasma and erythrocytes). Transit time flow probes (available through Transonic flowmeters, Ithaca, New York) are advantageous because they are easily calibrated and provide absolute flow values without the need for in situ calibration. However, Transonic flowsensors are temperature sensitive, and thus, need to be calibrated and temperature recordings compensated over the thermal range at which the sensors are used. Furthermore, the sensors are quite bulky, which restricts their use in confined spaces or when assessing flow in small specimens or vessels. For such applications, Doppler flowmeters may be more appropriate. According to the manufacturer, accurate flow recordings are also dependent on the ratio between probe size and vessel diameter, and thus, choosing the correct probe size for a given application is critical. The limited lead length of commercially available transit time flow probes constitutes another important limitation in experiments on aquatic organisms, especially when used in smaller vessels and/or test subjects, as lead length declines with smaller probe sizes. Finally, flow determinations using transit time flowmeters may be sensitive to shifts in probe positioning on the vessel, and thus, the probe must be properly sized and secured to avoid biased flow readings due to movement artifacts. The utilization of transit time flow probes has, since its conception in 1978, see (Drost 1978), generated a wealth of data on cardiac output (Cox 2010; Speers-Roesch et al. 2012; Eliason et al. 2013a, 2013b; Brijs et al. 2016b; Ekström et al. 2016a; Sandblom et al. 2016b), coronary blood flow (Davie and Franklin 1993; Cox et al. 2017; Ekström et al. 2017) and gastrointestinal blood flow (Eliason et al. 2008; Seth et al. 2010; Sandblom et al. 2012; Eliason and Farrell 2014; Brijs et al. 2018b) in numerous fish species.

X.2.6.6 Flow Probe Instrumentation of the Ventral Aorta, and Coronary and Celiacomesenteric Arteries

In many teleosts, the ventral aorta (VA) is accessible via an incision in the isthmus inside the opercular cavity (see **Box X.1 Case Study**). The VA is cleared by blunt dissection

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as not to damage the pericardium, surrounding tissues and nerves. In some species, one or several coronary arteries) traverse the VA before entering the pericardium, and thus, need to be separated from the VA. The VA may also be surrounded by a loose layer of connective tissue, which is dissected away. Once an ample section of the VA has been cleared, a flow probe can be fitted onto the vessel. The probe is then secured with a suture close to the cuff to minimize movement artifacts, after ensuring that an optimal signal has been attained. The probe lead is then secured with additional skin sutures inside the opercular cavity and along the body surface of the fish.

Certain teleosts, such as salmonids, scombrids and elasmobranchs have coronary arteries, which either originate from the branchial circulation (cranial supply) or the dorsal aorta (caudal supply) (Farrell et al. 2012). In at least some salmonids, a single coronary artery bifurcates from the hypobranchial artery (cranial supply) and is located in close proximity to the VA before entering the pericardium (Axelsson and Farrell 1993; Farrell et al. 2012; Ekström et al. 2017). After clearing the vessel with similar considerations as explained for the VA above, a probe can be placed around the artery downstream of the bifurcation to permit recording of coronary flow (Axelsson and Farrell 1993; Ekström et al. 2017). Alternatively, the coronary artery can be reached via a ventral incision to gain access to the vessel where it runs along the bulbus arteriosus or the VA inside the pericardium (Gamperl et al. 1994a, 1994b). However, this method requires opening the pericardium, which severely impairs cardiac function(s) (Farrell et al. 1988; Franklin and Davie 1993). If instrumenting fish with combined ventral and coronary aorticblood flow probes (e.g., Transonic) via the isthmus (Ekström et al. 2017), it may be advisable to access and instrument one vessel from each side (left/right) of the fish. Notably, Gamperl and colleagues (1994b) used a single custom-made Doppler probe integrating cuffs for both the ventral aorta and coronary artery. To date, only two studies have recorded coronary blood flow in elasmobranchs, and they employed similar approaches as explained above (Davie and Franklin 1993; Cox et al. 2017).

Blood perfusion of gastrointestinal tissues can be quantified by measuring blood flow through the celiacmesenteric artery. The celiacmesenteric artery branches from the dorsal aorta close to the efferent branchial arteries and often branches again to form the gastric and intestinal or mesenteric arteries, which perfuse the stomach and intestines (Farrell et al. 2001). In Rainbow Trout, the celiacmesenteric artery can be accessed via a ventro-dorsal incision starting from the right pectoral fin (Brijs et al. 2016a). Once an appropriate length of the vessel has been dissected free, a flow probe is fitted around the vessel downstream of the bifurcation from the dorsal aorta. The incision in the peritoneal cavity is then closed, preferably with interrupted stitches using monofilament sutures (e.g., 3.0 Prolene, Ethicon Inc., Somerville, New Jersey, USA) through both muscle and skin, which closes the wound and reduces the risk of infections.

X.2.6.7 Determinations of Regional Blood Flow by Intra-arterial Indicators

Blood flood distribution has also be quantified with intra-arterial injection of colored or radiolabeled indicators, which are distributed and accumulated in visceral tissues as they are trapped in the narrowing capillary beds. After euthanatizing the fish, distribution of colored indicators can be detected and quantified using spectrophotometric techniques (e.g., see Booth 1978; Kolok et al. 1993), or by quantifying the emitted radiation from radiolabeled indicators using a scintillation (ionizing radiation) or a gamma (α, β, or γ radiation) counter. The latter method was first used in fish research by Don Stevens who

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used ^{131}I labeled protein (albumin) to assess blood flow distribution in resting and exercised Rainbow Trout (Stevens 1968). The recorded quantities of indicator from the entire fish, or the total quantity of the injected indicator, is compared to the quantities detected in specific tissues and organs. Another common approach is the use of radiolabeled or colored microspheres to assess arterial blood flow to systemic and muscle tissues (red/white), and the integument (e.g., skin) of fish, under routine conditions and after specific stressors, e.g., in Rainbow Trout (Barron et al. 1987; Kolok et al. 1993; Taylor et al. 1996; Gerry and Ellerby 2014), Arctic Grayling *Thymallus articus* (Cameron 1975a) and Channel Catfish *Ictalurus punctatus* (Schultz et al. 1999). Moreover, Cameron (1975b) used this technique to assess blood flow distribution to the spongy and compact cardiac tissue layers in various teleost species. It is important to note that the microsphere method may underestimate regional blood flow (Barron et al. 1987; Bushnell et al. 1992; Kolok et al. 1993; Thorarensen et al. 1993). This may relate to entrapment issues, due to the fact that smaller microspheres traverse and escape entrapment in the capillaries. Another confounding factor is that the labeled indicator may settle in the syringe, needle or cannula, which needs to be accounted for (Kolok et al. 1993). Lastly, the site of injection will ultimately affect the distribution of the indicator to various tissues, and thus species-specific vascular morphology should be considered. All told, this technique should be used and interpreted with great caution (Farrell et al. 2001).

X.3 DATA AND OUTCOMES

The techniques detailed above yield numerous types of data and information for the researcher. Vascular casting provides information about the structure and perfusion of the organ system(s) of interest. Quantitative analysis of anatomical casts requires the use of software that can process three-dimensional data. Blood sampling can produce information about reproductive status, iono and osmoregulation disruption, the stress response, sex, oxygen carrying capacity, immune response and countless other variables. This information is commonly used to evaluate fish health or its response to environmental perturbation. Blood must be processed carefully and purposefully as parameters can change with temperature (e.g., pH, partial pressure of oxygen), and time (e.g., ions, hormones). Heart rate has been used to assess how quickly fish respond to stressors, as a proxy for metabolic rate, and as an indicator for thermal tolerance. Blood pressure and cardiac output are used to assess cardiac capacity, cardiac workload, and cardiac oxygen demand. Regional blood flow can offer information about the oxygen supply and demand of different tissues under various environmental (e.g., temperature, hypoxia) and biological (e.g., feeding, exercise) conditions. During these experiments, the same cardiovascular parameters are typically measured over time on the same individual fish, so the statistical analysis must incorporate this design (e.g., Repeated Measures analysis of variance (ANOVA) or a Linear Mixed model).

X.4 EMERGING IDEAS AND FUTURE DIRECTIONS

Ecologists and ecophysiologists have long sought to estimate the energetics and physiological status of animals in their natural environments (Elsner et al. 1964; Nagy et al. 1984;

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Ropert-Coudert and Wilson 2005; Cooke et al. 2016). To achieve this, it is necessary to measure physiological variables that relate to energetics in the field, rather than in controlled laboratory settings. Only via field physiological studies is it possible to truly appreciate how organisms perform and maintain homeostasis in their everyday life (Costa and Sinervo 2004; Nagy 2005; Ropert-Coudert and Wilson 2005). However, as described in this chapter, measuring cardiovascular responses in completely undisturbed animals is challenging as most physiological recording techniques require some level of invasive surgery and a physical connection between the animal and the recording equipment (Costa and Sinervo 2004; Ropert-Coudert and Wilson 2005; Axelsson et al. 2007; Gräns et al. 2009b). Fortunately, recent innovations and miniaturization of physiological sensors and batteries have resulted in a range of fully implantable biotelemetry systems and bio-loggers. These systems allow the recordings of a wide array of physiological variables in free-ranging animals including heart rate and electrocardiogram (ECG), blood pressure and flow, electromyogram (EMG), and electroencephalography (EEG) (Hinch et al. 2002; Cooke et al. 2004; Ropert-Coudert and Wilson 2005; Vyssotski et al. 2006; Axelsson et al. 2007; Gräns et al. 2009b; Clark et al. 2010). New technologies are continually being developed, and exciting emerging tools include improved implantable probes to measure blood variables such as oxygen, pH, CO_2, and lactate. This suite of emerging techniques will enable physiologists to better understand how cardiovascular function supports behavior and performance in the natural environment.

X.5 TERMS

Cardiac output (CO): volume of blood pumped from the heart per minute

Electrocardiogram (ECG): the measurement of the electrical activity during the cardiac cycle

Heart rate: the number of heart beats per minute

Hematocrit: volume percentage of red blood cells in the blood

Leucocrit: volume percentage of white blood cells in the blood

Mean cellular hemoglobin concentration (MCHC): the concentration of hemoglobin within a red blood cell

Oxygen consumption rate (MO_2): amount of oxygen consumed by the whole animal per unit of time

Pericardium: membrane enclosing the heart

Red blood cell (RBC) or erythrocyte: primary role is to transport oxygen in the blood

White blood cell (WBC) or leukocyte: primary role is immunity and cell defense

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X.6 REFERENCES

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