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COMMENT

Comment: Practices for Drawing Blood Samples from Teleost Fish

We read with interest a recent paper by Duman et al. (2019), which, based on the title, presumably was intended to serve as an overview of practices for drawing blood samples from teleosts. Given that the "go-to" reference for blood sampling of fish is Houston (1990), an update certainly seemed in order. However, as organismal biologists and stress physiologists, we were disappointed at the emphasis on the sampling of dead fish, with very limited discussion of live-sampling methods, and the focus on blood collection solely for assessing disease states. Indeed, the purpose of blood sampling is largely irrelevant in the context of a methods-oriented paper like this (e.g., the purpose could be to assess health, stress, genetics, oxygen carrying capacity, endocrine function, maturation status, etc.) except in cases where the collection method itself may perturb the parameter of interest (e.g., as in cortisol, glucose, and lactate titers; Lawrence et al. 2018).

Even while recognizing that the outlet for the work was an aquaculture journal, we were also disappointed by the seemingly narrow focus on fish in aquaria or culture facilities, especially given the broad title of the paper. Duman et al. (2019) draw upon a range of literature (including studies on wild fish) in creating their narrative, but they fail to make the connection between what they present and the broader application of blood sampling techniques in understanding general fish biology. This contrasts with what was presented by Houston (1990). Indeed, aside from methods like cannulation, which requires a more substantial surgical procedure (e.g., Clark et al. 2011), blood sampling can occur in a multitude of settings (e.g., riverbank, boat, aquaculture facility, reef crest, clinic, etc.) such that the source of the fish and the sampling location are largely irrelevant.

There are several aspects of Duman et al. (2019) that warrant specific critique. The first is the statement that the heart of a fish has "two ventricles": that is simply incorrect (e.g., Farrell and Smith 2017). This may seem trivial, but inexperienced phlebotomists attempting to obtain blood from cardiac puncture should recognize that there is indeed only a single ventricle. Relatedly, the authors repeatedly mention drawing blood from "veins." The reality is that at most locations in a fish's body (especially the caudal peduncle), the likelihood of obtaining a sample consisting entirely of venous blood (i.e., not a mixed sample with arterial blood) would be rather low (e.g., O'Neill et al. 1998; Mandelman and Skomal 2009; Esbaugh et al. 2016). On the surface, this may seem like a minor detail, but if one is measuring blood gases, for example, the knowledge of whether a blood sample is drawn from a vein or an artery is critically important (hence why in most studies one refers to sampling blood from the caudal vasculature).

The authors note that blood sampling is used infrequently on fish (especially ones in aquaria) because (1) the veins are not visible under the skin, (2) many fish species are small (<20 g), (3) there may be inadequate blood volume for sampling in aquarium fish, and (4) there may be a low chance of the fish surviving after blood is drawn. Duman et al. (2019) go on further to suggest that it is also not practical or feasible to conduct an average of two to three fish health examinations using blood withdrawal.

As mentioned above, blood can be drawn from veins and arteries, but there are very few instances in which the source matters (e.g., work on blood gases). Beyond that, there are common anatomical landmarks that enable one to draw blood irrespective of whether the vasculature is visible. Duman et al. (2019) suggest that to obtain blood from the caudal vasculature, it is important to avoid the fish's internal organs (e.g., gonads, intestines, air sac, etc.). In Figure 8, they show their proposed location for sampling the caudal vasculature—a location that we strongly advise against unless one has euthanized the fish prior to sampling. In the text, they suggest that the sampling should be done posterior to the anus, but Figure 8 shows otherwise (anterior). Blood collection in the location depicted on Figure 8 would be very difficult if not impossible without causing unnecessary damage to vital tissues and organs, which contrasts with the recommendations of the authors in the text. To our knowledge, blood is very rarely drawn from that location in a live fish, and the proposed sampling location was not discussed in the previous review by Houston (1990). Instead, sampling from the caudal vasculature is typically conducted quite posterior to the anus (see Figure 1; i.e., the caudal peduncle) and usually posterior to the anal fin (recognizing interspecific variation), which minimizes the risks of causing damage to vital organs. In one passage, Duman et al. (2019) suggest that the needle should be inserted 4–5 cm anterior to the tail, but this is poor guidance, as the distance will vary with body size and morphology. On some species (like

Common Carp *Cyprinus carpio* and Giant Trevally *Caranx ignobilis*), we have approached from a more lateral position (rather than ventrally—and much more laterally than depicted in Figure 9), but that is uncommon in practice. Moreover, the distance between the point of insertion and the vasculature is much shorter in the caudal peduncle, thus enabling one to use a shorter needle, which is easier to maneuver and less likely to become blocked or bent during phlebotomy. We also feel that the guidance regarding landmarking differently for fusiform versus compressiform fish may be misleading, and based on our sampling we have found generalizations to be difficult. The authors also suggest that the fish be held upright during blood sampling, which is again a rarity, but we recognize that it is an approach used by some researchers. This position is cumbersome and would usually require keeping the fish out of water (as in the linked video provided by Duman et al. 2019), causing undue stress in a live animal.

Despite Duman et al. (2019) suggesting that blood sampling is restricted to "large" fish, researchers have successfully drawn blood from even tiny fish, such as Zebrafish *Danio rerio* (Pedroso et al. 2012), including repeated samples (Zang et al. 2015). Some analytical techniques (e.g., transcriptomics and endocrinology) require relatively small quantities of blood for analysis such that repeated blood sampling is possible even from fish of small body sizes (Lewis et al. 2010; Cook et al. 2012). Duman et al. (2019) suggest that

> for biochemical parameters, such as the complete blood count, examination of liver and kidney enzymes, and analysis of specific hormones, researchers have found that on average, 5 mL of blood sample and 2 mL of serum sample are usually sufficient.

Such a statement is misleading and may cause many researchers to reconsider blood sampling given that 5 mL may not be practical or possible without causing negative impacts to the fish.

There are plenty of examples of fish having nonlethal blood samples drawn and surviving both over the short term and the long term. The authors claim that

> One of the important points of blood collection via caudal puncture is that the fish must be under mild anesthesia to reduce movement of the fish and the possible loss of vacuum.

We dispute this, as we have blood-sampled thousands of fish—representing numerous species and a diversity of settings—without anesthesia. In our experience, survival of unanesthetized fish after blood sampling is improved by minimizing the duration of sampling, air exposure, and associated handling stress. The method we use involves holding fish supine in a padded, water-filled, V-shaped trough (Cooke et al. 2005).

As is noted by Duman et al. (2019), it is possible for the movement of the fish to break the vacutainer's seal, thus prolonging sampling times. However, the benefit of not using general anesthesia is that the fish are not impaired upon release, which is a critical period for fish that are released into the wild. We are particularly concerned given that the paper by Duman et al. (2019) could be read by researchers and Institutional Animal Care and Use Committee members, who would be left to assume that blood sampling should only be performed on dead or heavily anesthetized fish. This itself is problematic, as such sampling procedures can influence the blood parameters of the fish, particularly if the blood is not sampled quickly from the animal (e.g., Houston et al. 1971; Oikari and Soivio 1975; Bourne 1984). Moreover, it could create unnecessary complications for researchers, who would need to justify to institutional administrators why it is reasonable to draw blood from fish without anesthetizing them.

An important message for researchers considering blood sampling is to first determine whether it is necessary to anesthetize or kill the fish to obtain samples. With adequate practice and refinement of procedures, it is possible to obtain nonlethal blood samples from the caudal vasculature without the use of anesthesia (Cooke et al. 2016). However, mild anesthesia can sometimes facilitate blood sampling during instances in which the fish proves unruly or the experience of the phlebotomist is lacking. Moreover, it is possible to repeatedly sample blood from individuals, with the interval being dependent upon the size of the fish and the size of the blood sample. The 3-month interval proposed by Duman et al. (2019) has no scientific basis: sometimes it may be on the order of minutes or hours, and in other cases it may be weeks or months depending on the nature of the research questions being asked. The effects of repeated blood sampling on parameters such as hematocrit can be offset by re-injecting saline-suspended red cells back into the animal (e.g., Rogers et al. 2003; Rodela et al. 2012; Zimmer and Wood 2014), thus making repeated sampling possible over short sampling intervals. However, applying a "rule" across the board is not helpful, especially considering the great diversity in fish size and physiology.

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STEVEN J. COOKE* AND MICHAEL J. LAWRENCE

Fish Ecology and Conservation Physiology Laboratory, Department of Biology and Institute of Environmental and Interdisciplinary Sciences,

Carleton University, 1125 Colonel By Drive, Ottawa, Ontario K1S 5B6, Canada

Graham D. Raby

Great Lakes Institute for Environmental Science, University of Windsor, 401 Sunset Avenue, Windsor, Ontario N9B 3P4, Canada

Amy K. Teffer

Department of Forest and Conservation Sciences, University of British Columbia, 2424 Main Mall, Vancouver, British Columbia V6T 1Z4, Canada

Ken M. Jeffries

Department of Biological Sciences, University of Manitoba, 50 Sifton Road, Winnipeg, Manitoba R3T 2N2, Canada

Andy J. Danylchuk

Department of Environmental Conservation, University of Massachusetts Amherst, 160 Holdsworth Way, Amherst, Massachusetts 01003, USA

Timothy D. Clark

School of Life and Environmental Sciences, Deakin University, 75 Pigdons Road, Geelong, Victoria 3216, Australia

*Corresponding author: steven.cooke@carleton.ca
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ORCID

Steven J. Cooke https://orcid.org/0000-0002-5407-0659
Amy K. Teffer https://orcid.org/0000-0001-5322-2617

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