Title: Function and control of the fish secondary vascular system, a contrast to mammalian lymphatic systems

Authors: J. L. Rummer[1,2*], S. Wang[1,3], J. F. Steffensen [4], and D. J. Randall[1,5]

Affiliations:

[1]Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong, S.A.R. China.

[2]ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Queensland, 4811, Australia.

[3]Fisheries College, Jimei University, 43 Yindou Road, Xiamen, 361021, China.

[4]Marine Biological Laboratory, University of Copenhagen, Strandpromenaden 5, Helsingor, DK-3000, Denmark.

[5]Department of Zoology, University of British Columbia, 6270 University Blvd., Vancouver, British Columbia, V6T 1Z4 CANADA.

*Correspondence to: jodie.rummer@jcu.edu.au

Summary: Teleost fishes and mammalian lineages diverged 400 million years ago, and environmental requirements (water vs. air) have resulted in marked differences in cardiovascular function between fish and mammals. Suggestions that the fish secondary vascular system (SVS) could be used as a model for the mammalian lymphatic system should be taken with caution. Despite molecular markers indicating similar genetic origin, functions of the SVS in teleost fish are probably different from those of the mammalian lymphatic system. We determined that, in resting glass catfish, (*Kryptopterus bicirrhis*), plasma moves

from the primary vascular system (PVS) to the SVS through small connecting vessels less than 10 μm in diameter, smaller than the red blood cells (RBCs). During and following hypoxia or exercise, flow increases, and RBCs enter the SVS, possibly via β-adrenoreceptor-mediated dilation of the connecting vessels. The volume of the SVS can be large and, as RBCs flow into the SVS, the haematocrit of the PVS falls by as much as 50% of the resting value. Possible functions of the SVS, including skin respiration, ionic and osmotic buffering, and reductions in heart work and RBC turnover, are discussed.

Key index words: lymphatic, secondary vascular system, stress

Abbreviations: ASR, aquatic surface respiration; DA, dorsal aorta; f_H, heart rate; ISO, isoproterenol; K, Fulton's condition factor; MS-222, tricaine methane sulfonate; PHT, Phentolamine; PROP, propranolol; PVS, primary vascular system; P_wO_2, water oxygen levels; RBC, red blood cell; SVS, secondary vascular system

Introduction:

The mammalian lymphatic system consists of blind-ending lymphatic vessels, lymph nodes, and other tissues, including the spleen, that collect, filter, and return fluid that drains from the tissues to the venous system and transport fats and proteins from the gut into the blood system (Alitalo et al., 2005; Wang and Oliver, 2010). In teleost fishes, some circulatory vessels contain clear fluid and have been described as lymphatic vessels (Kampmeier, 1969). Recently, Yaniv et al. (2006) and Isogai et al. (2009) also demonstrated that these vessels share some morphological and molecular characteristics with the lymphatic vessels found in other vertebrates, and suggested that these clear vessels in zebrafish could be used as a model for the mammalian lymphatic system. However, several decades ago these same vessels were hypothesized as part of a secondary vascular system (SVS), receiving inflow from the arterial system via arterio-arterial anastomoses and emptying into the venous circulation (Jensen et al., 2009; Steffensen et al., 1986; Steffensen and Lomholt, 1992; Vogel, 1981; Vogel, 1985; Vogel and Claviez, 1981). These vessels are not blind-ending and sometimes include red blood cells (RBCs) (Ishimatsu et al., 1992). Furthermore, there is no evidence that the SVS vessels are draining edematous tissues or playing a role in lipid transport. If not lymphatic, what then is the role of this secondary vascular system?

The volume of the SVS can range from 10-50% to nearly twice the volume of the primary circulation (Bushnell et al., 1998; Gallaugher and Farrell, 1998; Skov and Steffensen, 2003; Steffensen and Lomholt, 1992) and therefore may serve to buffer osmotic and ionic changes in the primary circulation. Under resting conditions, no RBCs are present in the SVS. As many of the capillaries of the SVS are superficial, it may be that plasma supplies nutrients to the skin, and gas transfer occurs between water and superficial structures directly across the skin surface (Steffensen and Lomholt, 1992). During exercise

the addition of RBCs to the SVS could augment cutaneous O_2 uptake. However, during aquatic hypoxia, there could be cutaneous loss of O_2 across the skin. We hypothesize, therefore, that exercise will be associated with RBC flow into the SVS, and aquatic hypoxia will shut down RBC flow into the SVS.

Glass catfish (*Kryptopterus bicirrhis*) were used to investigate the control mechanisms and functions of the SVS. Glass catfish are transparent, making non-invasive, *in vivo* observations and experiments possible. Furthermore, substantial baseline observations have been made on the primary (PVS) and secondary (SVS) vascular systems in this species (Jensen et al., 2009; Steffensen and Lomholt, 1992; Steffensen et al., 1986). Our objectives were to: 1) map flow patterns in the SVS using injected fluorescent microspheres and video-microscopy, 2) map SVS flow patterns without injecting microspheres in un-anesthetized fish during rest, exercise, and aquatic hypoxia – with and without surface access, 3) use pharmacological methods to further understand how SVS flow is regulated and 4) measure any changes in PVS haematocrit associated with the SVS filling with RBCs.

Materials and Methods

Experimental Animals

A total of 135 glass catfish (mean ±s.e.m. wet mass = 0.583 ±0.019 g; standard length = 47.6 ±0.5 mm) were transported to the Department of Biology and Chemistry at the City University of Hong Kong from a local supplier and maintained at 22 °C in dechlorinated municipal tap water under a natural photoperiod between the months of November 2010 and May 2011. Fish were fed daily to satiation with frozen bloodworms, but food was withheld for 24 h prior to experimentation. All animal care and experimental protocols comply with animal ethics regulations outlined by the Department of Biology and

Chemistry at the City University of Hong Kong.

Blood collection

The same protocol was used for collecting blood for all treatments. Fish were euthanized via head trauma, and the caudal fin was severed to bleed the caudal vein and artery into a heparinized microcapillary tube. This technique allowed the glass capillary tube to be placed in immediate contact with the caudal artery and vein, thus ensuring other fluids were not collected. Furthermore, fish capture and blood collections were done quickly (<20s) to minimize any effects that basic handling stress may have on the integrity of the RBCs. Samples (8-20 µl depending on the size of the fish) were centrifuged at 5000 rpm for 3 minutes and used to determine haematocrit.

Series 1: Flow patterns

It was first important to determine the size of RBCs during rest and whether size and shape would change if cells swelled during stress. This would permit us to select fluorescent microspheres to inject into the glass catfish PVS and SVS to track distribution and determine flow patterns. To determine RBC size, blood was collected from five fish and smeared onto glass slides with either a drop of Cortland's saline or one of two concentrations of a β-adrenergic agonist, Isoproterenol Hydrochloride (ISO; Sigma-Aldrich #15627), 0.001mM (prepared in Cortland's saline) (Caldwell et al., 2006). The RBCs were imaged using a fully motorized compound microscope (Olympus, model #BX61) with a Cool CCD still image camera (Olympus, model #DP-72) with capturing software (Image Pro Plus, v. 6.2). For each blood sample, 20 RBCs were randomly selected and measured using the tools integrated in the capturing software. Mean lengths for long and short axes and the ratio between the long and short axes were reported so that the appropriate size for

the fluorescent microspheres could be selected.

To determine blood flow patterns, fish were lightly anesthetized with tricaine methane sulfonate (MS-222) (1:25 diluted stock solution that was 4% w/v made using 1M Tris buffer at pH 9). Each fish was positioned on a moist sponge, left lateral side facing up and continuously moistened using a damp paper towel. Using an Olympus stereoscope (model #SZX12, Tokyo, Japan), the dorsal aorta (DA) of the fish was located, and a 10µL Hamilton ™ syringe (model #80330) with 33G 0.8" PT2 needle (model #7803-05) was positioned approximately 5mm posterior to the periphery of the operculum. A 2µl volume of fluorescent microspheres was slowly (over approximately 10 s) injected into the PVS of the fish via the DA. Four different sized microspheres, 0.02 µm (green), 1 µm (blue), 4 µm (green), and 10 µm (red) (Invitrogen, #F8787, #F8815, #F8859, and #F8834 respectively) were used. Prior to injection, aliquots were sonicated for 5 min. and then centrifuged at 3,000 x g for 3 min. to reduce agglomeration, as per manufacturer recommendations.

Immediately upon injection, fish were transferred to a plastic petri dish filled with approximately 20 ml water without anaesthetic, enough to completely cover the fish, which was not restrained but remained calm in the petri dish. We observed the fish using fluorescent light (X-cite Series 120Q EXFO using GFP/UV/RFP filters) and videos of the injection site were recorded to trace distribution of the microspheres (Video camera: Panasonic GPUS932HE; Software: Aver Mediacenter v 1.7.3, H727 PCIe Hybrid DVBT HDMI) until no further changes could be observed.

Series 2: Regulation of flow

Fish were transferred from holding tanks using dip nets and held individually, one per 500ml glass beaker, in continuously aerated, clean water maintained at 22 °C for 2 h before experiments commenced. During this time, all beakers were placed in white

polystyrene containers with lids so fish would stay light in colour; dark background results in melanophore dispersal mediated by cholinergic, muscarinic-type receptors (Fujii et al., 1982) making fish less transparent and harder to visualize. This was also done to ensure fish were in an unstressed state so valid resting conditions could be observed (see below). Additionally, because it was determined from earlier observations that anaesthetic promoted RBC distribution into the SVS fish were not anaesthetized for Series 2 experiments so that other regulatory mechanism could be revealed.

Resting

These fish remain quiet when handled carefully and gently, but stressed glass catfish have RBCs in the SVS. If we transferred a fish to the petri plate for observation without RBCs entering the SVS, we referred to this fish as "resting". Because of subsequent experimental design we also determined that multiple transfers, if done gently, did not promote RBC entry into the SVS. For resting measurements, un-anaesthetized individual fish (n=28) were placed in plastic petri plates containing 20 ml of well-aerated, clean water and examined under a dissecting microscope using white light, similarly to how fish were observed using fluorescent light, as described above. The caudal fin region of the fish was examined for the presence of RBCs in the SVS. Next, by gently tilting the petri plate to the side, the fish was positioned (ventral side up) against the sidewall of the petri plate. Because of the transparent nature of this species, the heart was also visible to the naked eye. Therefore, the image of the beating heart was projected live onto a computer monitor and heart rate (f_H) was visually assessed and recorded as the number of beats per minute (using 20s assessments). After each count, the caudal fin and posterior ventral regions of the fish were re-examined for presence or absence of RBCs in the SVS. This was replicated three times in order to achieve a mean f_H per fish and form a consensus as to the status of RBCs

in the SVS. Videos were recorded for each individual.

Blood was collected from another group of resting fish (n=10) and used to determine resting haematocrit. Fish were then gently pat dry, weighed (g), measured (standard length, mm), and length-to-weight ratio (Fulton's condition factor, $K = (W \bullet 100) \bullet L^{-3}$) was calculated.

Hypoxia exposure

Hypoxic conditions were created in a 30-l white polystyrene container either allowing the fish to use aquatic surface respiration (ASR) or directly covering the surface with a clear Perspex sheet to prevent the fish from using ASR. Nitrogen was bubbled into the water, which reduced water oxygen levels (P_wO_2) to 20 mmHg. Hypoxic conditions were continuously monitored using a pre-calibrated fiber optic O_2 sensor (PreSens, Precision Sensing GmbH, Regensburg, Germany) (tip diameters 50-140 μm) amplified using an Oxy-micro oxygen meter and signal amplifier (PreSens, Precision Sensing GmbH, Regensburg, Germany). Data were collected throughout the duration of each experiment at a sampling rate of 1 Hz, and integrated with the manufacturer's software packages for PC Windows. All data were saved as text files and analyzed using Acqknowledge® Data Acquisition Software (Version 3.7.3, BIOPAC Systems, Inc.).

We used four groups of fish, each for f_H and RBC observation in hypoxia either permitted (group 1, n=7) or not permitted to access the surface (group 2, n=8), and for blood sampling immediately (group 3, n=10) or 1 h (group 4, n=10) following exposure to hypoxia with no access to surface. All fish were observed (as described above) prior to hypoxia experiments to ensure they were exhibiting resting conditions in terms of f_H and the absence of RBCs in SVS. Hypoxia exposure (P_wO_2 = 20.3±0.1 mmHg) lasted for 5min. during which time they were either permitted surface access where they were observed

191 performing aquatic surface respiration (ASR) or not permitted surface access due to a clear
192 Perspex lid on the surface of the water. For groups 1 and 2, following hypoxia exposure,
193 fish were immediately (within 10s) observed and data collected for f_H and the status of
194 RBCs in SVS. Fish were then allowed to recover for 1h (1h-recovery) in well-aerated,
195 clean water and monitored again. We were not able to monitor all fish immediately and so
196 the n values for the immediate and after 1h time points are different. Fish were permitted to
197 recover overnight (12h), after which time survival was assessed and fish were returned to
198 holding facilities and not used for further experimentation. For this and all subsequent
199 experiments, fish that did not survive 12h following treatment were not included in
200 analyses. Fish from group 3 exposed to hypoxia (no ASR), and after 5 min. removed from
201 treatment. Blood was immediately collected from each fish to determine haematocrit, and
202 fish were weighed and measured as described above. Fish from group 4 were exposed to
203 hypoxia (no ASR) in the same manner, and after 5 min. removed from treatment, allowed
204 1h-recovery in well-aerated, clean water. Then blood was sampled from each fish to
205 determine haematocrit and fish were weighed and measured as described above.
206
207 *Exercise*
208 We used three groups of fish, each for f_H and RBC observation immediately and
209 then 1h following exercise (group 1, n=10) and for blood sampling immediately (group 2,
210 n=9) or 1 h (group 4, n=11) following exercise. First, all fish were observed (as described
211 above) prior to exercise to ensure resting conditions. Exercise was simulated by chasing
212 individual fish for 30s with a plastic probe in a beaker of well-aerated, clean water.
213 Following this, fish were permitted recovery for 30s during which time they were
214 transferred to either the microscope, blood sampling station, or well-aerated, clean water for
215 1h recovery. Blood was sampled to determine haematocrit and fish were weighed and

measured as described above. The fish that were observed for f_H and RBCs in the SVS were then permitted to recover overnight (12 h), after which time survival was assessed and fish were returned to holding facilities and not used for further experimentation.

Pharmacological experiments

All fish for the pharmacology experiments were observed (as described above) prior to treatments to ensure resting conditions. Then, each fish was exposed to one of the following treatments in well-aerated, clean water for 5 min. (n=7-12 for each group): β-agonist, Isoproterenol Hydrochloride (ISO; Sigma-Aldrich #15627) at a final concentration of 1 mM (Caldwell et al., 2006; Rummer and Brauner, 2011; Rummer et al., 2010), β-antagonist, Propranolol Hydrochloride (PROP; Sigma-Aldrich #P0884) at a final concentration of 2 mM (Caldwell et al., 2006; Rummer and Brauner, 2011; Rummer et al., 2010), or α-antagonist, Phentolamine (PHT; Sigma-Aldrich #P7547) at a final concentration of 0.01 mM (Kagstrom and Holmgren, 1997; Namoto and Yamada, 1987). Immediately following 5 min., exposed fish were observed as described for resting conditions and transferred back to well-aerated, clean water. In the case of PROP, treatment fish were observed and subsequently exposed to the agonist (ISO) for 5 min., observed (PROP+ISO) and then transferred back to well-aerated, clean water. Following either single or double (sequential) exposure to drugs, fish were allowed 1 h-recovery in well-aerated, clean water. Following 1 h-recovery, measurements were taken for ISO and PROP+ISO treated fish and observations made as described above. Finally, fish were permitted to recover overnight (12 h), after which time survival was assessed and fish were returned to holding facilities and not used for further experimentation. All drugs were dissolved in well-aerated, clean water from the same source used for maintaining fish in the laboratory. All solutions were made fresh for each experiment.

Statistical analyses

Data are presented as mean ± s.e.m. unless otherwise stated. All P_wO_2 data were saved as text files and analyzed using Acqknowledge® Data Acquisition Software (Version 3.7.3, BIOPAC Systems, Inc.). All data were compared statistically between treatments, and statistical differences were detected via paired t-test, ANOVA, or repeated measures ANOVA and, when necessary, a post-hoc Holm-Sidak multiple comparisons test. All statistical analyses were conducted using SigmaStat for Windows 3.5.0.54 (Systat Software, Inc., 2006), and all analyses were interpreted using $\alpha < 0.05$ to determine statistical significance.

Results

RBC size and dispersal

Fluorescent microspheres were injected into the PVS. The 0.02, 1 and 4 μm microspheres dispersed rapidly throughout both the PVS and SVS with a clear image for the 0.02 and 4 μm microspheres (Fig. 1). The 10μm fluorescent microspheres gave a strong signal but did not disperse from the dorsal aorta (DA), the original injection site. The RBCs obtained from a resting, unstressed glass catfish have a mean length of 11.5±0.5 μm, width of 7.1±0.12 μm, and a width to length ratio of 63.0±0.1% (means ± s.e.m., n=6). When the RBCs increased in volume, the cells became more rounded and less flattened. When exposed to 1μM isoproterenol, RBC width to length ratio increased significantly from 63.0% to 69.3±0.7% ($p<0.001$, paired t-test). We measured several red (10μm) fluorescent beads and found them spherical with diameters between 10 and 1μm.

Flow in the secondary vascular system

From preliminary observations on un-anaesthetized fish we determined that under resting conditions only plasma flows slowly through the SVS. Flow in the tail section is always towards the head away from the tail and caudal heart. When a fish became stressed (by injection, removing scales, temperature change, excessive handling), RBCs rapidly entered the SVS. We categorized flow in the SVS into four stages (Fig. 2). Stage 0, resting, was when no RBCs could be observed anywhere in the SVS (i.e. only plasma). Stage 1 was when a few non-moving RBCs could be observed in the SVS. Stage 2 was when RBCs could be observed entering the SVS and flow, albeit slow, was noticeable. Stage 3 was if flow was rapid and RBCs were densely packed throughout the SVS.

Experimental exposure

We observed a total of 5 mortalities throughout the entire project with no association with any particular treatment or time period.

Hypoxia and exercise

Hypoxia and exercise both increased flow and number of RBCs in the SVS, and this resulted in a marked reduction in hematocrit in the PVS ($p < 0.001$, ANOVA; Fig. 3). Flow of RBCs into the SVS was more pronounced when fish were permitted to use aquatic surface respiration (ASR) throughout the duration of hypoxia exposure (Fig. 4). Immediately following hypoxia exposure with no ASR, RBCs in the SVS were sparse and flow was minimal or absent (Fig. 4). Without surface access, fish exposed to hypoxic water swam to the bottom of the experimental container, even resting on its side in some instances. This behaviour clearly contrasted that of the actively swimming hypoxia-exposed fish that were permitted surface access. After 1h recovery, there was greater influx of RBCs into the SVS in both hypoxic fish groups. However, flow of RBCs into the SVS

was more pronounced in exercised fish when compared to hypoxia-exposed fish (Fig. 4). Exercised fish also exhibited a significant increase in f_H that started to come back to resting by 1h but was still significantly elevated ($p < 0.001$, RM ANOVA, Fig. S1). However, there was no change in f_H in fish exposed to hypoxia regardless of surface access (ASR) ($p = 0.115$, RM ANOVA, Fig. S1). Neither hypoxia nor exercise was associated with any change in the length-to-weight ratio (Fulton's condition factor, $K = (W \cdot 100) \cdot L^{-3}$, ANOVA; Fig. 3).

Pharmacological experiments

Exposure to the β-agonist, isoproterenol (ISO), dramatically increased RBC numbers and flow into the SVS. These changes were associated with an immediate increase in f_H but recovery by 1h ($p < 0.001$, RM ANOVA, Fig. S1). This response was less pronounced when compared to the significant increase in f_H with exercise, which persisted even after 1h recovery ($p < 0.001$, RM ANOVA, Fig. S1). The effects of ISO were ameliorated when exposure was combined with the β-antagonist, propranolol (PROP) (Fig. 4), which also significantly decreased f_H ($p < 0.001$, RM ANOVA, Fig. S1). Recovery was evident by 1h. Upon exposure to the α-antagonist (phentolamine; PHT), however, RBC flow into the SVS was minimal, and there was no effect on f_H (Fig. S1).

Discussion

There have been several reports of arterio-arterial anastomoses between the SVS and PVS in fish (reviewed by Steffensen and Lomholt, 1992) using vascular castes and *in vivo* microscopic observation. During resting conditions, only plasma flows through the SVS, presumably because inflow diameters are too small to allow RBC entry. Fluorescent microspheres, 0.02, 1, and 4 μm were small enough to pass into the SVS within seconds

(Fig. 1), but 10 µm microspheres were not, indicating that input vessels from the PVS to the SVS had a diameter of less than 10 µm. Hypoxia, exercise and stress resulted in varying levels of RBC flow into the SVS. We hypothesized that RBC entry into the SVS would be shut down during aquatic hypoxia to prevent oxygen loss to the water across the skin. This was largely the case when fish were exposed to hypoxia but prevented from accessing the surface to perform ASR. However, fish actively swimming at the surface using ASR during hypoxia exposure exhibited some RBCs and flow into the SVS. Therefore we cannot fully accept our hypothesis that the SVS is not in use during hypoxia. We also did not measure pressure and do not know if the opening of the anastomoses during hypoxia and exercise in the glass catfish was due to increased PVS arterial pressure or relaxation of presumed sphincters around the anastomoses. Stresses such as hypoxia and exercise have been reported to increase circulating catecholamines in fish (Caldwell et al., 2006; Primmett et al., 1986; Thomas and Perry, 1992), and this, combined with our results outlining the effects of adrenergic manipulations indicate that catecholamines are involved in the increased RBC entry into the SVS following hypoxia and exercise. Nitric oxide is also reported to mediate SVS dilation under hypoxia (Jensen et al., 2009) but was not investigated here.

Increased RBC flow into the SVS markedly decreased PVS hematocrit following both hypoxia and exercise in glass catfish. We realize the difficulty of blood collection without admixture in such small fish. However, the procedure used was the same for all fish and we see consistent differences between groups. It is unlikely that admixture occurred in only one group or to a different extent between groups, but this remains a possibility. The fish itself did not change volume, as indicated by no change to body condition factor, a proxy used to indicate changes in body volume. Therefore, water influx is unlikely to play a substantial role in the observed hematocrit change. PVS haemoconcentration due to blood volume reduction (Pearson and Stevens, 1991;

Yamamoto, 1988), RBC swelling (Caldwell et al., 2006), and splenic release (Gallaugher et al., 1995) occurred in fish during exercise and hypoxia and may have offset the large decrease in PVS haematocrit, i.e. our measurements may underestimate the effect of increased RBC flow into the SVS on the haematocrit of the PVS. However, the magnitude of the hematocrit reduction we observed indicates that the volume of the SVS is at least equal to, if not larger than that of the PVS in glass catfish. We know of no other report of a reduction in Hct during hypoxia or exercise in fish. Although, Steinhausen et al., (2008) reported that sockeye salmon exhibited no significant change in Hct with exercise. In fact hypoxia and exercise have been reported to increase Hct in fish (Gallaugher and Farrell, 1998; Wells, 2009). These differences in the magnitude of the changes in Hct with exercise and hypoxia may be related to the volume of the SVS. It has been reported that the secondary vascular system can range from 10-50% to nearly twice the volume of the primary circulation (Bushnell et al., 1998; Gallaugher and Farrell, 1998; Skov and Steffensen, 2003; Steffensen and Lomholt, 1992). The magnitude of other effects, such as splenic release of RBCs, increases in RBC volume and blood volume reduction (see above) may also play a role in differences in the magnitude of the Hct change with increased RBCs flow into the SVS of different fish species.

Various hypotheses as to the function of the SVS have been proposed. We suggest that retaining RBCs in a small PVS volume in resting fish will reduce work of the heart and RBC production. Recently, Rasmussan and colleagues investigated the role of the SVS in rainbow trout and concluded that, because the SVS can regulate concentration and distribution of cell types, e.g. immune cells, the SVS likely plays an important role in immune function (Rasmussen et al., in press). Ishimatsu and colleagues suggested the SVS plays a role in acid-base regulation and epithelial ion transport probably via Cl^-/HCO_3^- exchangers, which may also be superficially located (Ishimatsu et al., 1992). There are

large fluid shifts with as much as 20% of PVS plasma volume moving into the tissues during exercise or exposure to hypoxia (Pearson and Stevens, 1991; Yamamoto et al., 1983; Yamamoto, 1988). Thus, increased SVS flow and, therefore, more rapid mixing of the SVS and PVS could buffer ionic or osmotic changes in the PVS during exercise or other forms of stress. When only plasma flows through the SVS, the probable function is to deliver nutrients and remove waste (Steffensen and Lomholt, 1992), with gas transfer occurring directly across the skin. Flushing the SVS with RBCs following exercise would enhance O_2 uptake across the skin. The SVS was less flushed with RBCs during aquatic hypoxia (no ASR) than it was when fish were in normoxic water during recovery from hypoxia or both immediately following and upon 1h recovery from exercise. However, these changes were small and not reflected in differences in the reduction in PVS haematocrit between hypoxic and exercise exposed groups.

The SVS is restricted to and variable amongst the Actinopterygii and related to function rather than phylogenetic position (Skov and Bennett, 2004). A more extensive SVS may be linked to increased scope for activity, which in turn may be associated with enhanced skin O_2 uptake via the SVS during exercise. There is no evidence of an SVS in lungfish (Vogel and Mattheus, 1998), but whether our Silurian vertebrate ancestors had a SVS subsequently lost in the Acanthodii or the SVS evolved in the Actinopterygii is unknown. What is clear is that the organization of circulation and requirements for fluid balance are different in aquatic and terrestrial vertebrates. It follows that the changes in the vertebrate circulation between extant fish and mammals are to be expected. Tissue edema and fluid drainage can be problematic in terrestrial mammals, as they have high blood pressure and are subject to the effects of gravity that come with living in an aerial environment. While there is a paucity of information on the mechanisms underlying tissue fluid balance in fish, fish blood pressures are lower than those of mammals, and living in

water eliminates the effects of gravity because the density of the environment is similar to that of their own body. Furthermore, there is no evidence that the SVS plays a role in countering tissue edema and aiding fluid drainage. The SVS is not associated with the celiac, mesenteric or renal arteries, and no association has been made between increased SVS flow and food intake (Skov and Steffensen, unpublished observations reported in Skov and Bennett (2004)). The SVS, unlike the lymphatic system of mammals (Kayama and Iijima, 1976; Robinson and Mead, 1973), is not involved in uptake from the gut. Therefore, it is clear that the SVS is not functioning in the same way as the mammalian lymphatic system. Additionally, the SVS vessels are not blind-ending, and flow originates directly from the primary vascular system. Following hypoxia and exercise, RBCs enter the SVS and flow increases. Thus, the presence of molecular markers for the mammalian lymphatic system in the SVS vessels cannot be used as indicators of lymphatic function in fish. In summary, and in contrast to the conclusions of Yaniv et al. (2006), the SVS and the PVS are not distinct vascular systems, and there are circumstances during which the SVS does contain RBCs. We conclude, and agree with recent arguments by Vogel (2010), that the SVS has different form and function from, and is not a suitable model for, the mammalian lymphatic system.

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Figure Captions:

Fig. 1. Fluorescent microspheres dispersing through the caudal region of the glass catfish secondary vascular system (SVS). Microspheres that were 0.02μm (green, panel A) and 4μm (green, panel B) were small enough to pass into the SVS. A white box on panel A is enlarged to represent the body segment illustrated in panel B. CH, caudal heart.

Fig 2. Light microscope images of the ventral-most vessel of the glass catfish secondary vascular system (SVS). In all images, the top of the panel represents the dorsal side of the fish, bottom is ventral, right is posterior, and left is anterior. An arrow in each panel indicates the ventral most-vessel of the SVS and typical direction of flow. Each stage (0-3) of red blood cell (RBC) dispersal into the SVS is depicted in separate panels. Stage 0: There are no RBCs in the SVS, Stage 1: Few RBCs in the SVS, little or no flow, Stage 2: RBCs starting to enter the SVS, individual cells can still be identified, flow is noticeable but slow, Stage 3: Rapid flow of densely packed RBCs through the SVS.

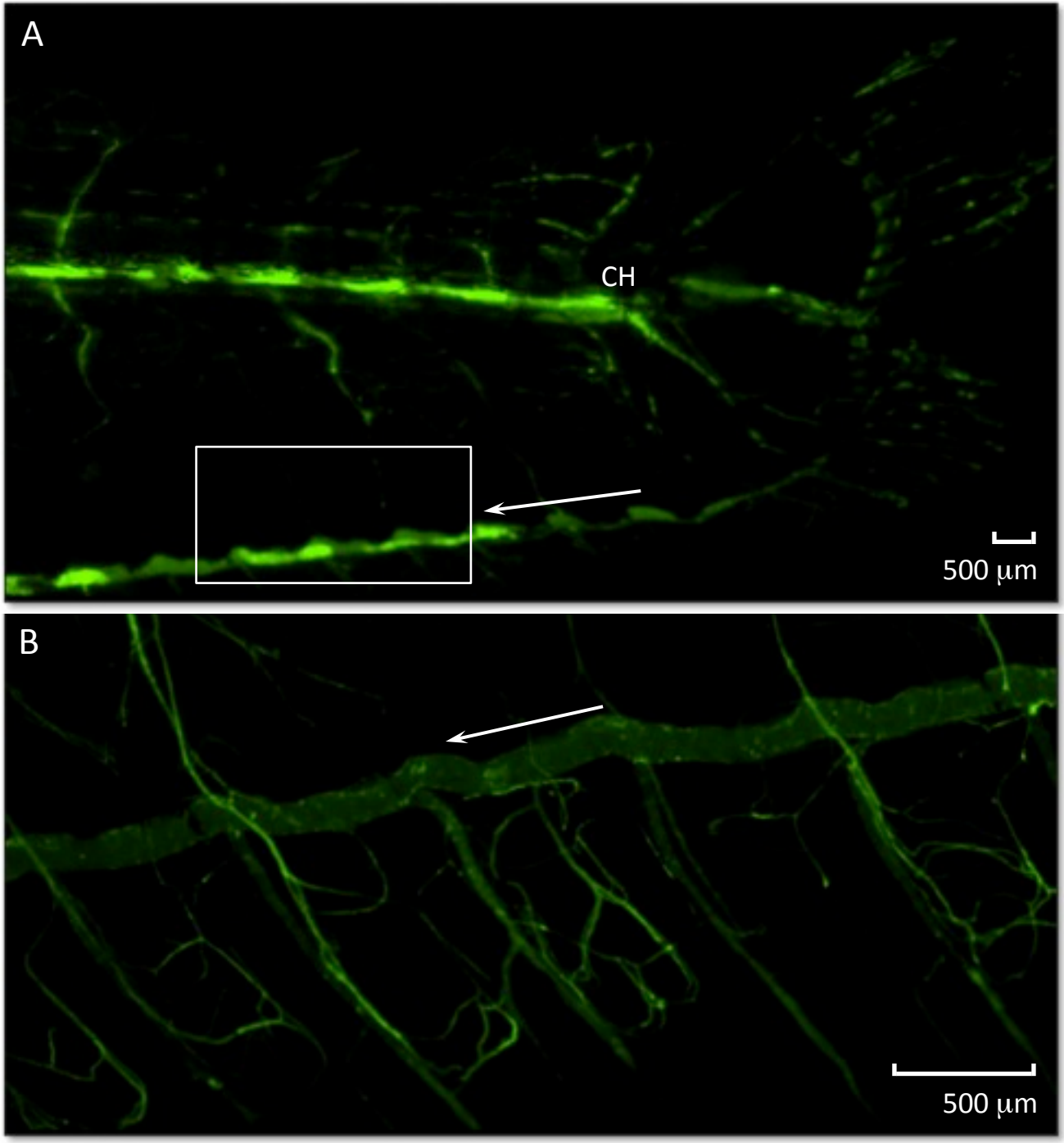
Fig. 3. Changes in haematocrit and condition factor in glass catfish upon and following exposure to hypoxia (no ASR) and exercise. Data are plotted as means and standard errors. Haematocrit was measured at rest, immediately following treatment, and following 1h recovery. A different group of fish was used for each treatment, i.e. not repeated measures. The number of replicates (n) are noted for each sampling time, and different letters demarcate significant differences within treatment (ANOVA). Inset shows no change in the fish condition factor ($K = (W \bullet 100) \bullet L^{-3}$) at each sampling.

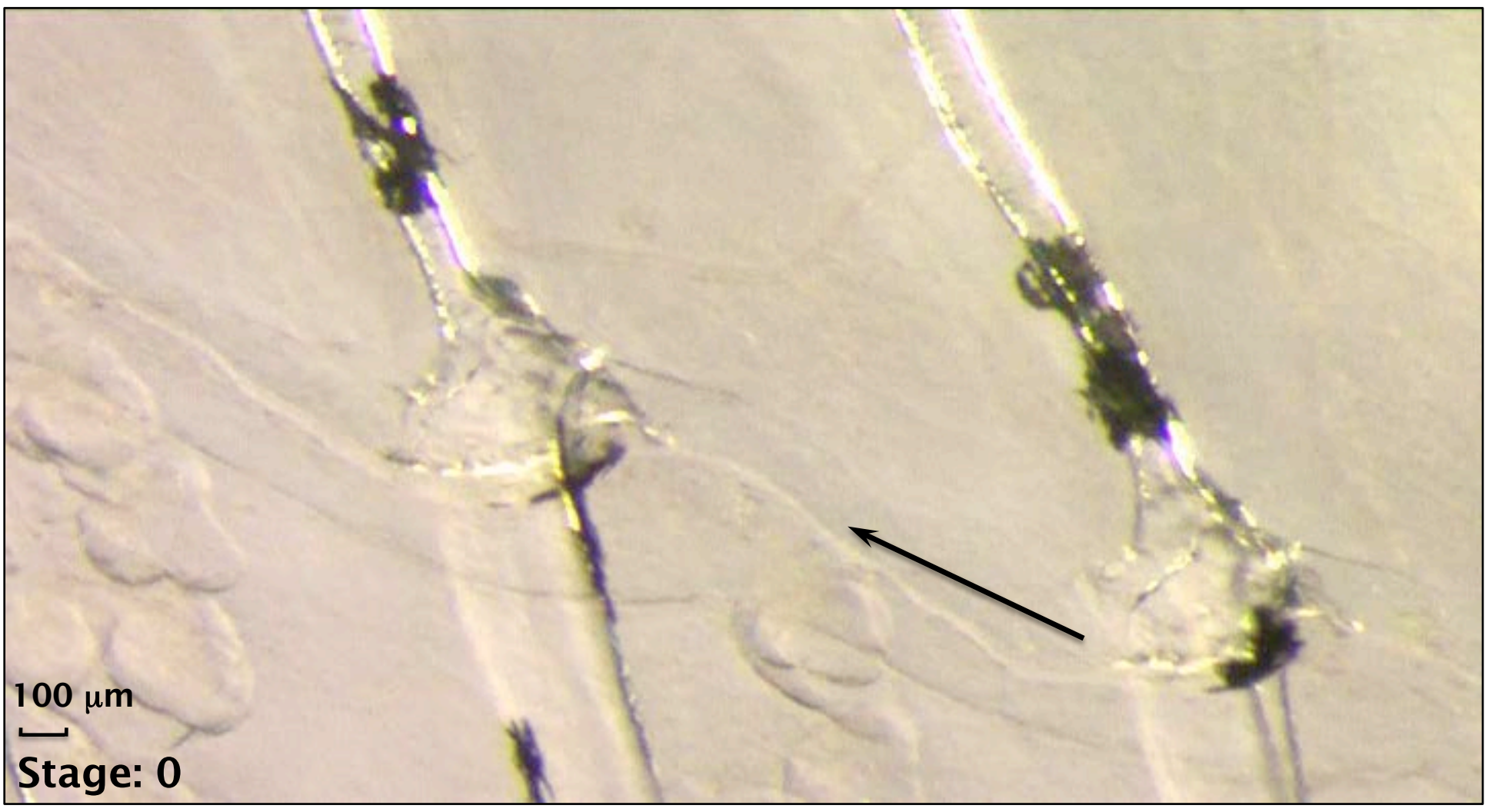
Fig. 4. Changes in secondary vascular system (SVS) flow patterns by stage (0-3). See Fig. 2

for stage descriptions. The stage of the SVS was observed immediately (*Imm.*) or 1h following exposure (*1h*). The y-axis represents the percentage of fish observed exhibiting each stage of the SVS within a treatment group (x-axis). The number of replicates (n) are noted on the top of each bar for each treatment. Red blood cells, RBCs; aquatic surface respiration, ASR; propranolol, PROP; isoproterenol, ISO; phentolamine, PHT.

Supplemental Materials

Fig. S1. Changes in heart rate (f_H) in glass catfish upon and following exposure to hypoxia with surface access (ASR) and without surface access (no ASR) immediately and following 1h recovery (panel A), immediately following and 1h into recovery from exercise (panel B), and upon treatment with isoproterenol (ISO), propranolol (PROP), both sequentially (ISO+PROP), or phentolamine (PHT) immediately or following 1h recovery (panel C). Data are plotted as means and standard errors. A different group of fish was used for each type of treatment. Different letters demarcate significant differences within each panel, and p-values are noted at the upper, right corner of each panel (ANOVA). See Materials and Methods for further details.


A
CH
500 μm
B
500 μm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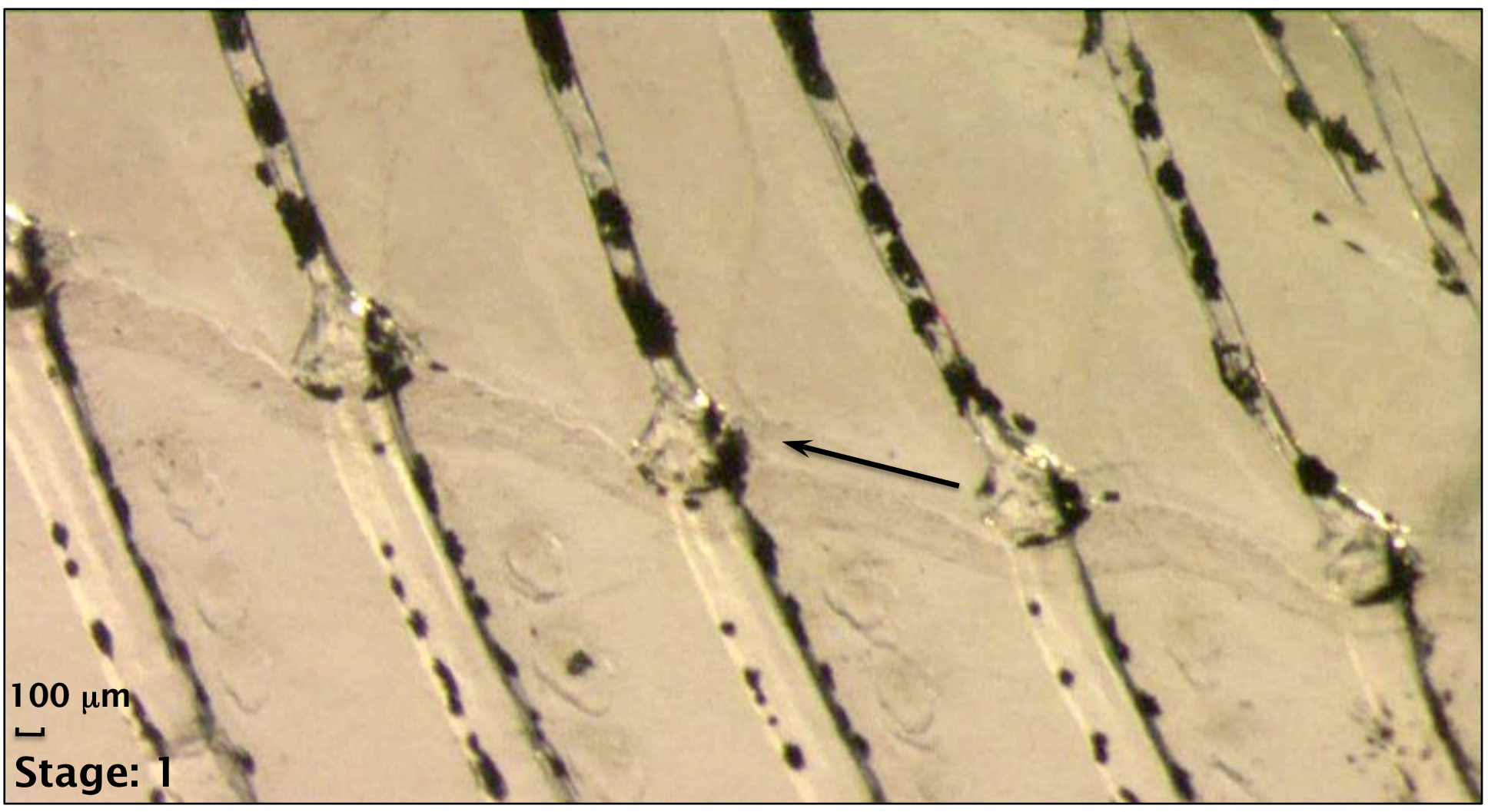
Stage: 0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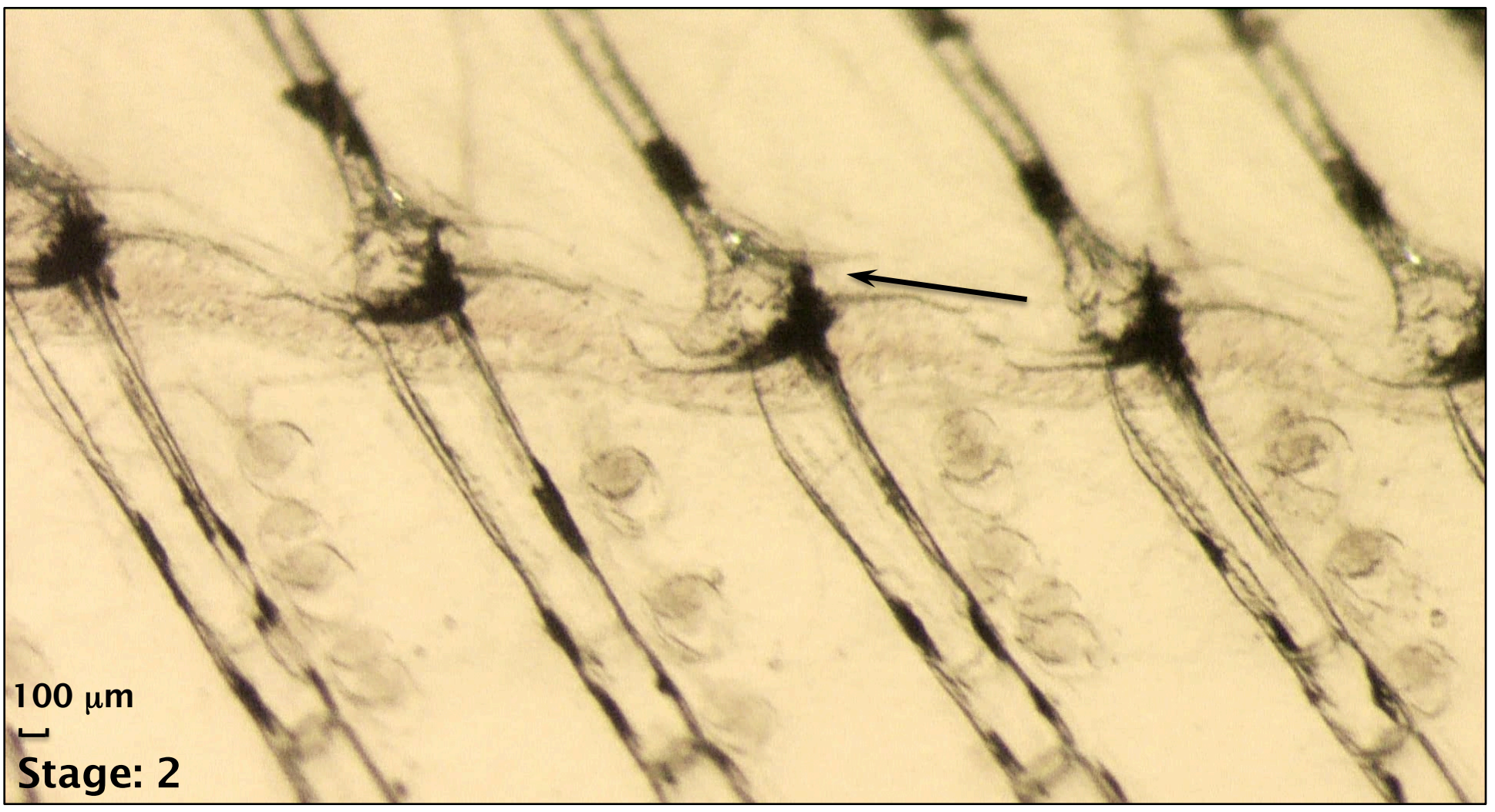
Stage: 1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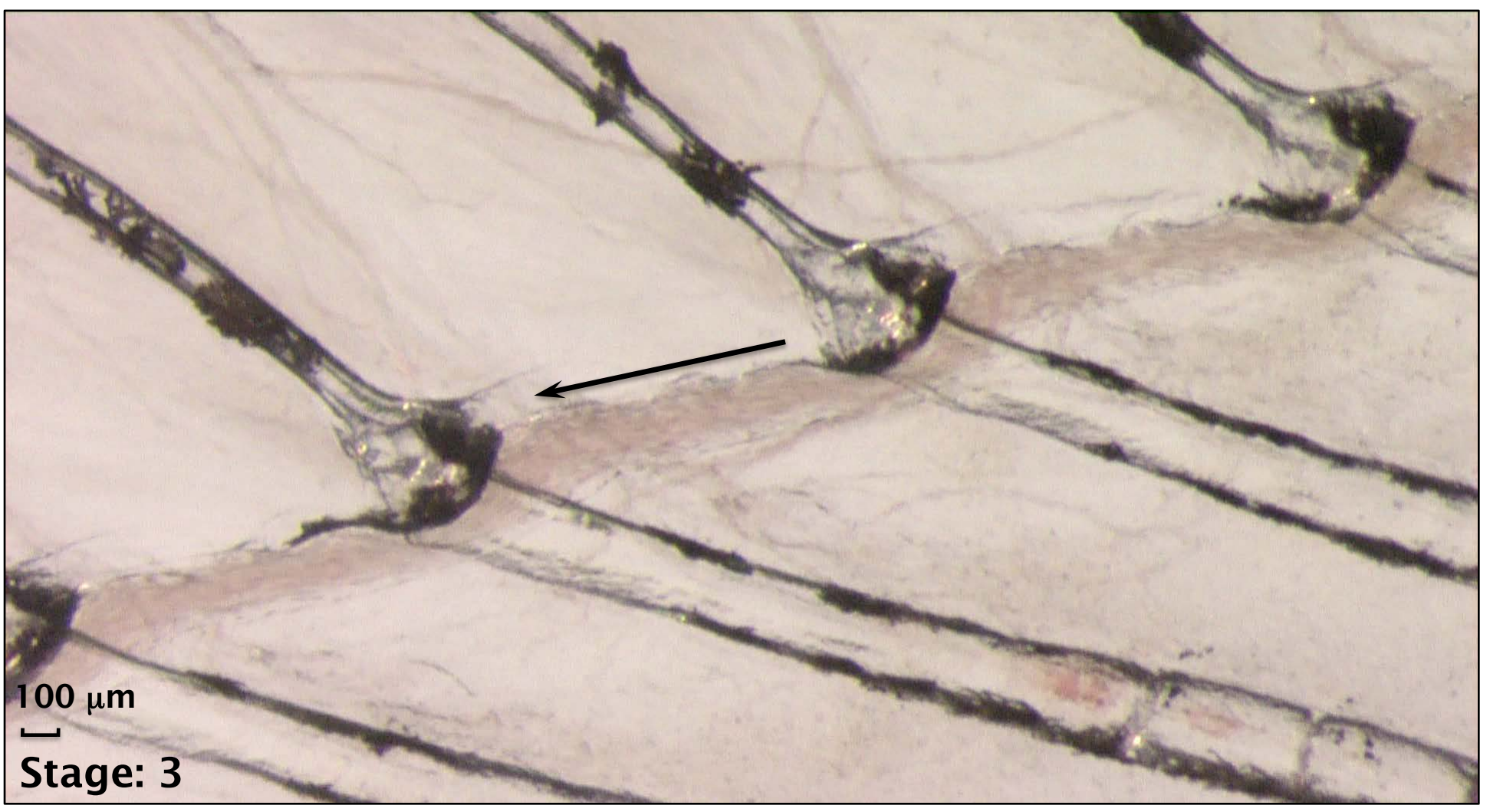
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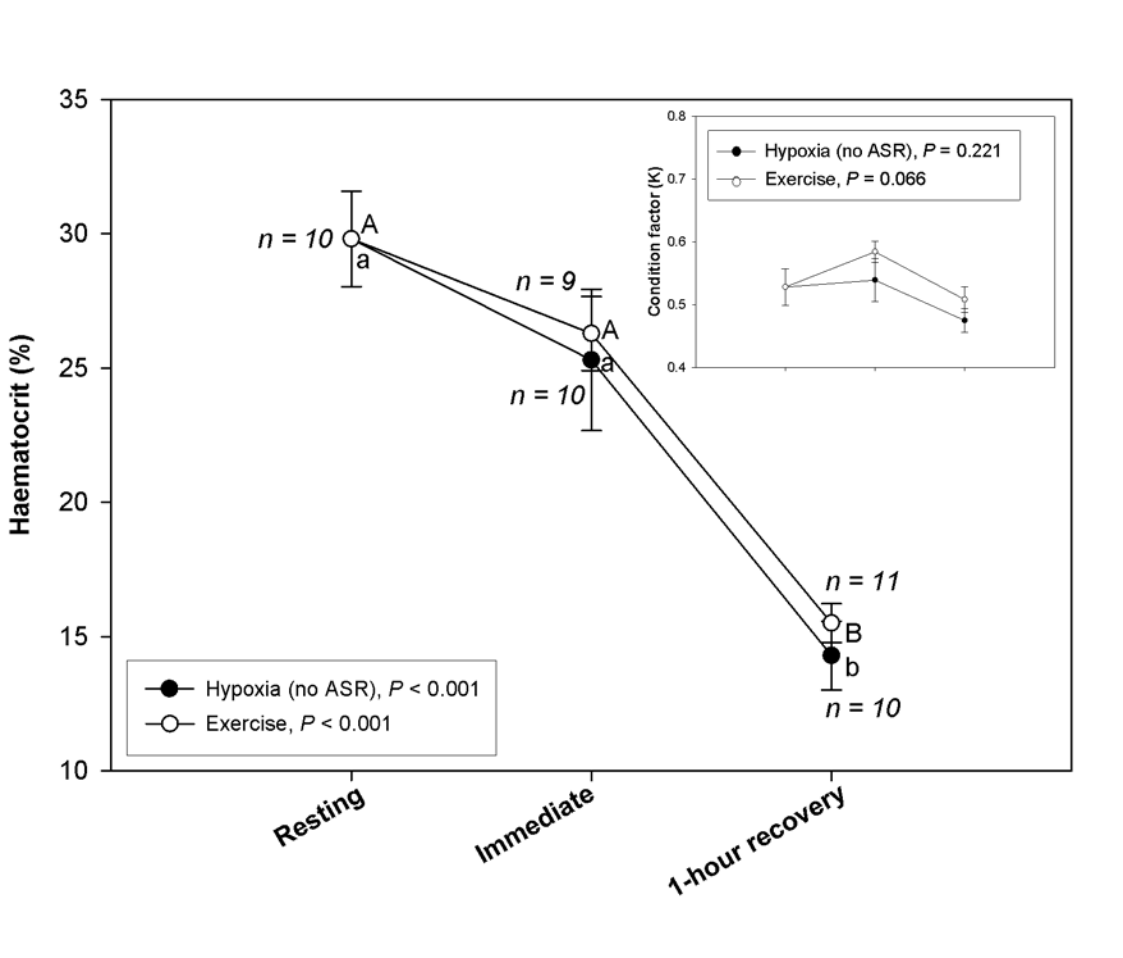
Stage: 3


Haematocrit (%)
35
30
25
20
15
10
Resting
Immediate
1-hour recovery
n = 10
A
a
n = 9
A
a
n = 10
n = 11
B
b
n = 10
Hypoxia (no ASR), P < 0.001
Exercise, P < 0.001
Condition factor (K)
0.8
0.7
0.6
0.5
0.4
Hypoxia (no ASR), P = 0.221
Exercise, P = 0.066