

## TECHNIQUES FOR PHYSIOLOGY

# Techniques for the *in vivo* assessment of cardio-renal function in zebrafish (*Danio rerio*) larvae

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## Key points

- The anatomical structures and discrete nephric regions of the pronephric zebrafish kidney are similar to those of the mammalian nephron.
- Renal blood supply and subsequent renal filtration rate are both key determinants of kidney function.
- Our study developed classical functional assays for larval zebrafish that effectively model the effects of the nephrotoxin gentamicin and high salt loading.
- The techniques demonstrate the disruption in ion and water balance resulting from reduced cardiovascular flow and renal filtration rate.
- The ability to measure cardio-renal function in zebrafish offers advantages associated with the fish model to gain a wider understanding of kidney physiology.

**Abstract** Zebrafish, a well-established vertebrate model, offer unique advantages for assessing renal function and physiology. Assays determining renal glomerular function based on cardiovascular erythrocyte flow and reduction of injected FITC-inulin were developed, each validated using the nephrotoxin gentamicin. Bland–Atlman analysis showed a strong association between measurements of the rate of inulin excretion and that of fluorescent reduction from the arterial vasculature. Reduced renal clearance of inulin, resulting from gentamicin or NaCl loading, was concurrent with reduced erythrocyte velocity, and yolk sac and pericardium oedema. These techniques, assessing pronephric function, highlight the potential for *in vivo* physiological study in this genetically tractable model.

(Received 11 November 2011; accepted after revision 7 February 2012; first published online 13 February 2012)

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**Abbreviations** CW, conditioned water; dpf, days post-fertilisation; FITC, fluorescein isothiocyanate; GFR, glomerular filtration rate; hpf, hours post-fertilisation; MS-222, tricaine methanesulfonate; PTU, 1-phenyl-2-thiourea; SNGFR, single nephron glomerular filtration rate.

## Introduction

The zebrafish (*Danio rerio*), with its prodigious breeding capabilities, *in vitro* development, and abundance of mammalian orthologous genes, has become a powerful vertebrate genetic model. The combination of advantageous biological traits and well established forward and reverse genetic techniques make the zebrafish well suited for the study of renal physiology and disease. The anatomically simple pronephric kidney of the translucent zebrafish larvae, comprising a single fused glomerulus and two nephrons lying alongside the posterior cardinal veins (Drummond, 2003), is fully functional by 3.5 days post-fertilisation (dpf) (Kramer-Zucker *et al.* 2005). Much of the complexity of the mammalian kidney is present in the zebrafish pronephros, and the eight discrete regions of the mammalian nephron are identifiable by 24 h post-fertilisation (hpf) (Wingert *et al.* 2007). With tractable genetics, the development of physiological techniques in the zebrafish is attractive; indeed we have previously developed approaches for assessing cardiac function (Denvir *et al.* 2008). Such techniques in zebrafish larvae will benefit the study of drug discovery, organogenesis, disease and cardio-renal physiology in all species.

The gold standard for measurement of glomerular filtration rate (GFR) is the clearance rate of inulin, a non-metabolised polysaccharide freely filtered but neither re-absorbed nor secreted by the nephron. During the first 2 days of embryonic development in zebrafish, ion and water regulation principally occurs at the skin ionocytes. Following the onset of filtration at ~48 hpf, the pronephros becomes an increasingly important osmoregulatory site (Drummond, 2003). Renal function is influenced by both arterial pressure supplying the kidney and the subsequent flow rate of filtrate through the glomerulus and nephron. Despite this, renal clearance in zebrafish larvae has not been determined in conjunction with measures of cardiovascular flow. Additionally, current measures of renal clearance in zebrafish larvae are known to be affected by artefacts (Hentschel *et al.* 2007). The approach of this study was to develop satisfactory renal clearance assays in the zebrafish larvae and determine influences of cardiovascular flow to assess the suitability of the zebrafish pronephric kidney for the study of renal physiology. The nephrotoxin gentamicin was used for assay validation and the model tested physiologically by determining responses to altered ambient salinity.

## Methods

### Ethical approval

All experiments were approved by the local ethics committee and conducted in accordance with the Animals

(Scientific Procedures) Act 1986 in a UK Home Office approved establishment.

### Zebrafish culture

The wild-type WIK line was used for all experiments; unless otherwise stated embryos/larvae were maintained at 28.5°C in 55 ml conditioned water (CW). Larvae were anaesthetised with 40 µg ml<sup>-1</sup> MS-222 (tricaine methanesulfonate) in CW. All experimental procedures were performed at room temperature, 23 ± 0.5°C.

### Gentamicin injection

Gentamicin was administered as described by Hentschel *et al.* (2005). Anaesthetised larvae (76 hpf) were injected, via the anterior common cardinal vein, with 2 nl of 5 mg ml<sup>-1</sup> gentamicin solution (as similarly used by Hentschel *et al.* 2005), containing 2.5% (w/v) fluorescein isothiocyanate (FITC)-inulin in saline as a marker of injection success; gentamicin was replaced with the vehicle in controls.

### Clearance assays

Five per cent (w/v) FITC-inulin was dissolved in 0.9% NaCl and dialysed with 1000 Da dialysis tubing for the removal of any unbound FITC. Anaesthetised larvae (100 hpf) were injected with 2 nl of FITC-inulin solution via the cardiac sinus venous, immediately following which the vasculature network could clearly be observed. Injected larvae that showed residence of FITC in the pericardial or yolk sacs immediately following injection were considered unsuccessfully injected and discarded.

**Clearance of intensity from the caudal artery.** After a 15 min post-injection recovery, larvae were placed on 1% agar and initial FITC intensity determined by imaging laterally over the caudal region (Fig. 1A) using a Leica MZ16F dissection microscope and Leica 300 FX camera under UV light with 480 nm excitation and 510 nm barrier filters. Larvae were then immediately transferred to 70 µl CW. After 4 h, larvae were removed and FITC intensity re-imaged. ImageJ software (National Institutes of Health) was used to determine three mean intensity values for each fish; mean percentage intensity change was determined to allow for variations in initial FITC intensity.

**Excretion of inulin into surrounding medium.** As a reciprocal approach in the gentamicin study, we determined the excretion of FITC-inulin into 70 µl of CW ('surrounding medium') over a 4 h period, between 100 and 104 hpf. FITC intensity was determined

**Table 1. Treatment protocol of conditioned water (CW), dilute medium (1/20th CW) and sodium supplemented CW**

Treatment	Marine salts (mg l <sup>-1</sup> )†	Supplemented NaCl (g l <sup>-1</sup> )	Final sodium concentration (mM)	pH
Control (CW)	60	0	0.748	7.4
Dilute medium (1/20th CW)	3	0	0.037	7.6
CW + supplemental NaCl				
CW + 10× Na <sup>+</sup>	60	0.40	7.57	7.3
CW + 25× Na <sup>+</sup>	60	1.00	17.8	7.3

†Marine salts (Tropic Marin®). Measured Na<sup>+</sup> in conditioned water 17.2 mg l<sup>-1</sup>.

spectrophotometrically (Tecan, Infinite M1000) using an excitation of 485 nm and emission of 538 nm.

### Exposure to low salinity medium and increasing sodium concentrations

Low salinity and altered sodium chloride solutions were prepared with deionised water as outlined in Table 1. Measured Na<sup>+</sup> in CW was determined by a BWB-2 flame photometer (BWB Technologies, Halstead, UK). From one cell stage to 24 hpf embryos were maintained in CW, after which embryos were transferred to treatment solutions in triplicate ( $n = 25$ ) and replenished daily by 50% with corresponding solution (all at 28.5 °C).

### Venous erythrocyte velocity: frame by frame analysis of single erythrocytes

Anaesthetised larvae were immobilised in 0.6% agar, flushed with CW, and given a 60 min recovery period. Erythrocyte velocity was determined from video capture and velocity analysis in the posterior cardinal vein. For gentamicin effects velocity was measured using a Zeiss Axioskop 2 compound microscope with an IonOptix Inc. camera at 29 frames s<sup>-1</sup>. This method proved inadequate for higher velocities observed in salinity-treated larvae, which were measured with a Leica MZ16F microscope and DFC 300 FX camera with Leica LAS video software between 45.3 and 46.7 frames s<sup>-1</sup>. Distance travelled by four erythrocytes per fish was determined using ImageJ over consecutive frames and mean velocity ( $\mu\text{m s}^{-1}$ ) measured.

### Transversal histological sections

Larvae were fixed in 10% formalin and prepared for histology as described by Sabaliauskas *et al.* (2006).

Resulting blocks of agarose containing zebrafish were then sectioned and oriented to enable transversal sectioning.

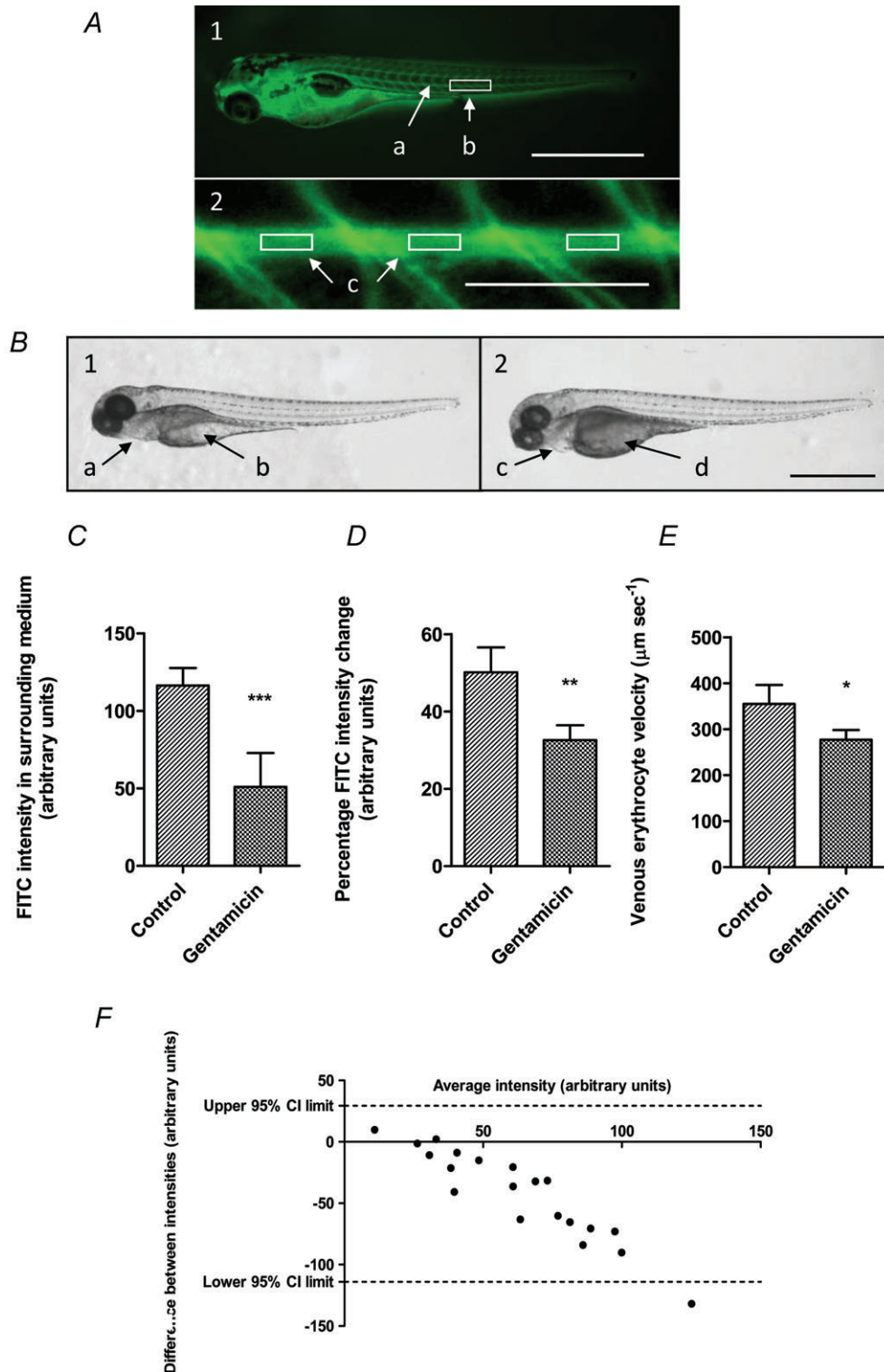
### Statistical analysis

Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Differences between gentamicin and control larvae were analysed with Student's unpaired *t* test. Data from both clearance rate assays were combined and compared by the Bland–Altman test. Differences between salinity treatment groups were modelled with one-way ANOVA and *post hoc* comparisons (against controls) modelled using Dunnett's multiple comparisons test. *P* values <0.05 were considered significant and means all reported with a standard error of the mean (SEM).

### Results

Measurements of FITC-inulin intensity over the caudal artery and excreted FITC-inulin successfully modelled the effects of the nephrotoxin gentamicin (Fig. 1) and altered ion composition (Fig. 2) in zebrafish larvae. Using a similar (2.3 nl 5 mg ml<sup>-1</sup>) 24 h dose in zebrafish larvae, Hentschel *et al.* (2005), reported several morphological and physiological changes associated with gentamicin toxicity including, brush border flattening, lysosomal phospholipidosis, the accumulation of tubular debris and reduced renal clearance. Unlike the mammalian kidney, renal flow in the zebrafish pronephros relies on motile cilia (Kramer-Zucker *et al.* 2005); consequently, any brush border flattening is expected to result in a reduced renal flow. Injected FITC-inulin was visible within the larval vascular network (100 hpf) after 30–60 s; the caudal artery providing a suitable area for instantaneous FITC intensity analysis (Fig. 1A). Larvae injected with gentamicin at 76 hpf developed gross phenotypes associated with oedema by 100 hpf; no such phenotypes were observed in control fish (Fig. 1B). Twenty-four hours post-gentamicin injection (100 hpf) reductions in renal clearance were detected by determination of both excreted FITC-inulin and FITC-inulin reduction in the caudal artery (Fig. 1C and D). Concurrent with reduced renal flow in gentamicin treated larvae was a reduced venous erythrocyte velocity (Fig. 1E). A Bland–Altman comparison of combined renal clearance data shows a good agreement between techniques (Fig. 1F).

All larvae subjected to high NaCl from 24 hpf developed pericardial sac oedemas, and darkened and distended yolks by 100 hpf; no such phenotypes occurred in other treatments (Fig. 2A). Further histological examination of the CW + 25× Na<sup>+</sup> group revealed intracranial, pericardial, and coelomic oedemas. Neural fissures and disarray of yolk droplets by increased intercellular



**Figure 1. Clearance assay validation – response of renal clearance and cardiovascular flow to gentamicin at 100 hpf**

A, larvae injected (100 hpf) with 2 nl 5% FITC-inulin. 1, FITC-inulin visible throughout vascular network 30–60 s post-injection (scale bar 1 mm); a, FITC intensity visible within caudal artery; b, marked area posterior of cloaca shows area used between somites 16 and 18 for FITC intensity analysis. 2, FITC intensity over caudal artery; c, intensity measured mid-artery distant from vessel edges between intersegmental vessels in three separate regions (scale bar 500  $\mu\text{m}$ ). B, larvae injected with 2 nl (5 mg ml<sup>-1</sup>) gentamicin (vehicle in controls) at 76 hpf;

spacing were also noted (Fig. 2B). This presentation of widespread oedema strongly suggests that ion homeostasis was compromised. Parallel with the oedema in larvae maintained in CW + 25× Na<sup>+</sup> were reductions in both renal clearance (Fig. 2C) and circulatory flow (Fig. 2D). Although exposure to the dilute medium had no measurable effect on renal clearance (Fig. 2C), circulatory flow was slightly reduced in comparison to controls, albeit to a lesser extent than larvae maintained in CW + 25× Na<sup>+</sup> (Fig. 2D). No measurable effects were observed in control and CW + 10× Na<sup>+</sup> groups. As renal function is sensitive to pH, potential effects from the slight variations in pH between salinity groups cannot be discounted.

The assessment of both renal and vascular flow revealed a relationship between cardiovascular and tubular flow in the pronephros; reduced renal flow was consistently associated with reduced cardiovascular flow (Figs. 1C–E and 2C and D). Due to its relatively simple anatomy, tubular flow may not act independently to arterial flow in the zebrafish pronephros. The failure of renal function and resulting oedema associated with gentamicin toxicity (Fig. 1C and D) and chronic high NaCl loading (Fig. 2C) demonstrates the necessity of renal flow in addition to the population of epithelial ionocytes for the maintenance of larval osmotic homeostasis at 4 dpf. These data indicate a positive feedback loop linking renal flow, vascular flow, oedema, and ion regulation/water balance as suggested by Hill *et al.* (2004). This may become irreversible once established and might explain the common oedematous phenotypes and associated yolk sac pathologies that result in larval fish subjected to a variety of insults. Indeed, oedema *per se* may also reduce renal clearance by physical disruption of the kidney structures (Hill *et al.* 2004).

Venous velocity was determined, as opposed to aortic velocity, as non-pulsatory venous flow allows frame by frame analysis. Maximum mean erythrocyte velocity (100 hpf) was reduced in gentamicin experiments (355  $\mu\text{m s}^{-1}$ ) compared to salinity and NaCl experiments (642  $\mu\text{m s}^{-1}$ ) and was associated with slower and faster renal clearances, respectively. This is expected to have been due to the additional injection at 76 hpf required for gentamicin administration. We have shown that a 2 nl injection volume, as used in this and previous studies for the administration of gentamicin and inulin, reduces venous velocity by 17.5% 30 min post-injection compared to fish punctured but not injected (S. A. Rider,

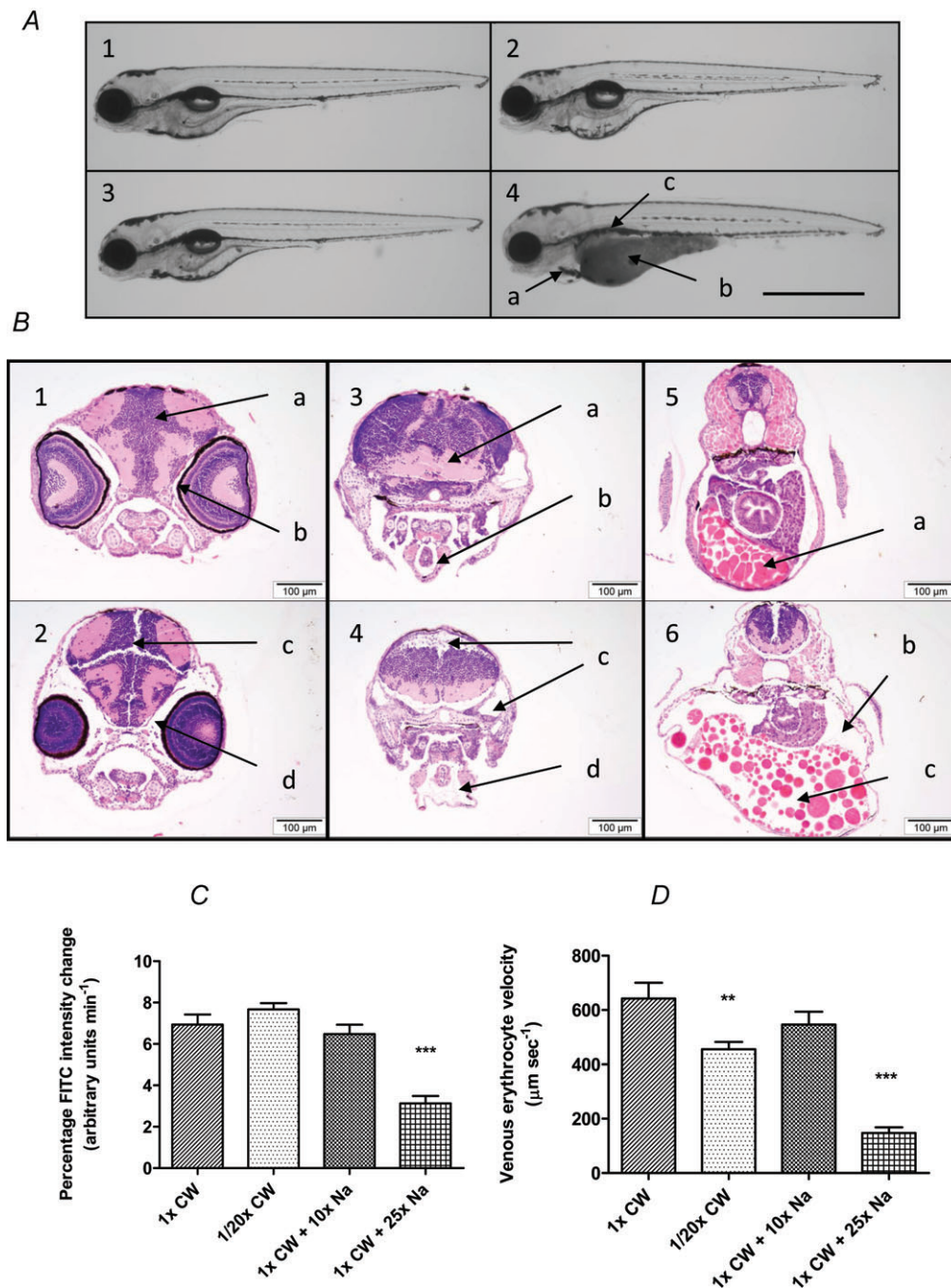
unpublished data). Due to the reduction in normal circulatory flow, induced volume expansion may also affect true renal clearance. Erythrocyte velocities and renal clearances here reported are for larvae maintained at 23 ± 0.5°C. Although within normal physiological ranges for this poikilothermic organism, erythrocyte velocities and clearance rates will be higher in fish maintained at 28.5°C; heart rates are 136 bpm at 22°C and 276 bpm at 28°C (Denvir *et al.* 2008).

## Discussion

The two techniques devised for the determination of renal clearance, using FITC-inulin, effectively modelled renal flow in the mature pronephric kidney. Determination of FITC intensity in the caudal artery has several advantages. Baseline concentrations of actual injected inulin are quantified allowing for normalisation; the relationship between inulin decay and initial concentration may not be linear. The caudal artery, which is easily visualised without 1-phenyl-2-thiourea (PTU) de-pigmentation, is of physiological relevance as the pronephros receives its blood supply from the dorsal aorta (Drummond, 2003). Furthermore, the technique is not affected by trapped FITC close to the injection site, as occurs with heart intensity measurements (Hentschel *et al.* 2005, 2007), which are unreliable, particularly with low molecular mass molecules, as true intensity in the heart is obscured by FITC leakage into the pericardial sac.

In mammals, ion and water homeostasis principally occurs by modulation of ion transport in the nephron and changes in GFR are typically transitory responses (Vallon, 2003). This may also hold true in the stenohaline larval zebrafish, which in response to varying salinities modulate endocrine genes including, *atrial natriuretic peptide (anp)*, *renin*, *prolactin*, *growth hormone (gh)* and *parathyroid hormone 1 (pth1)* (Hoshijima & Hirose, 2007). Given the oedematous morphology of larvae subjected to the chronic high NaCl loading, the associated reduced clearance rates and venous velocities are expected to be the result of pathological effects rather than homeostatic physiological responses. In adult euryhaline teleosts, total GFR in low and high salinity environments is increased or decreased, respectively, by regulation of

gross phenotypes (100 hpf) in all larvae (scale bar 1 mm). 1, control; normal pericardial (a) and yolk sacs (b). 2, gentamicin; oedema of pericardial sacs (c) and darkened and distended yolk sacs (d). C–F, further 2 nl injection of 5% FITC-inulin at 100 hpf. C, mean ( $n = 10$ ) intensity of FITC-inulin excreted into surrounding medium over 4 h significantly reduced ( $***P = 0.0004$ ) by gentamicin (116.3 arbitrary intensity units) versus controls (51.0). D, mean ( $n = 10$ ) percentage FITC-inulin intensity decrease from caudal artery over 4 h, significantly decreased ( $**P = 0.0019$ ) by 17.5% 24 h post-gentamicin injection. E, mean ( $n = 20$ ) posterior cardinal vein erythrocyte velocity significantly reduced ( $*P = 0.0118$ ) from 354.7 to 277.0  $\mu\text{m s}^{-1}$  by gentamicin. F, Bland–Altman analysis shows good agreement between FITC intensity change in the caudal artery and surrounding medium.



**Figure 2. Response of renal clearance and cardiovascular flow to salinity and sodium challenge at 100 hpf**

Larvae maintained in CW from one cell stage to 24 hpf prior to transfer to 1/20th CW, CW + 10 $\times$  Na<sup>+</sup>, CW + 25 $\times$  Na<sup>+</sup>, or maintained in CW. *A*, gross phenotypes in all larvae (100 hpf); 1, CW; 2, 1/20th CW; 3, CW + 10 $\times$  Na<sup>+</sup>; 4, CW + 25 $\times$  Na<sup>+</sup>. For these pericardial oedema (*a*), darkened and distended yolk sacs (*b*), and uninflated swim bladders (*c*) were observed (scale bar 1 mm). *B*, transverse sections ( $n = 9$ ): 1, control: normal neural tissue (*a*) and periocular space (9/9) (*b*). 2, CW + 25 $\times$  Na<sup>+</sup>: neural fissures (6/9) (*c*) and pericardial oedema (9/9) (*d*). 3, control: normal intracranial space (*a*) and pericardial sac (*b*). 4, CW + 25 $\times$  Na<sup>+</sup>: intracranial oedema (2/9) (*c*) and pericardial oedema (9/9) (*d*). 5, control: normal coelomic cavity and yolk (*a*). 6, CW + 25 $\times$  Na<sup>+</sup>: intracoelomic oedema (*b*), disarray of yolk globules with increased intercellular spacing (6/9) (*c*) (scale bars 100  $\mu$ m). *C*, mean ( $n = 20$ ) percentage FITC clearance in the caudal artery; only significantly reduced renal clearance *versus* controls in larvae maintained in CW + 25 $\times$  Na<sup>+</sup> ( $***P < 0.05$ ). *D*, mean ( $n = 9$ ) venous erythrocyte velocity; only significantly reduced by 1/20th CW ( $**P < 0.05$ ), 455  $\mu$ m s<sup>-1</sup>, and CW + 25 $\times$  Na<sup>+</sup> ( $***P < 0.05$ ), 116.3  $\mu$ m s<sup>-1</sup>, compared to controls.

single nephron glomerular filtration rate (SNGFR) and numbers of actively filtering glomeruli (Brown *et al.* 1978), filtration itself being a major determinant of urinary excretion.

With the possibility of high throughput analysis, tractable genetics, optical clarity, ease of manipulation and relatively low cost, the zebrafish model affords many exciting possibilities for the study of renal physiology. As a precursor to investigations of renal electrolyte transport the development of quantitative functional assays is key. The methods developed in our study modelled both cardiovascular and renal flows in the larval zebrafish, demonstrating its suitability for the study of functional renal physiology as well as molecular approaches.

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## Author contributions

All experiments were conducted at the University of Edinburgh Centre for Cardiovascular Science. All authors contributed to study conception and design, interpretation of data, and manuscript preparation and revision. Data were collected by S.A.R., J.dP. and K.N.R. There are no author conflicts of interest to disclose.

## Acknowledgements

We duly acknowledge support from the British Heart Foundation Centre of Research Excellence Award. J.J.M. and M.A.B. acknowledge the support, respectively, of Wellcome Trust Principal and Intermediate Research Fellowships.