Urine composition in pigs is regulated in both the ureter and the bladder

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Short title: Urine composition regulation in pigs

Summary

The former perception of the urothelium as an impermeable barrier has been revised during the last decade, as increasing evidence of changes in urine composition during its passage of the urinary tract has been presented. Since differences in urothelial

permeability between upper and lower urinary tract have been found, our aim is to demonstrate whether changes in urine composition occur during passage through the ureter.

We studied consecutive urine samples from both renal pelvises in six pigs and compared them to samples from the bladder and distal ureter. We further sampled urine during storage in the bladder at a fixed volume. All samples were analysed by measuring osmolality and pH, along with the concentration of the following parameters: Na^+, K^+, Cl^-, creatinine, urea.

Urine alkalinity increased significantly during passage of the ureter. Creatinine concentration, pH and K^+ increased significantly during the passage from pelvis to the bladder. All other parameters increased non-significantly during the passage to the bladder. The increase in concentration was more pronounced at low concentrations in the pelvis. During storage in the bladder, there was a significant increase by in urea concentration.

Changes in the composition of urine occur during its passage from the renal pelvis to the bladder and during storage in the bladder. Despite the brief transit time, significant changes in alkalinity were found already during passage through the ureter.

Introduction

During the last decade increasing attention has been given to the various functions of the urothelium (Khandelwal *et al.* 2009, Birder and Andersson 2013, Lasič *et al.* 2015). Tight junctions between urothelial cells and lining of the luminal urothelial surface by uroplakins and glycans work together in reducing urothelial permeability (Riedel *et al.* 2005). This accounts for the historical perception of the urothelium acting as the "skin" of the urinary tract allowing nothing to pass (Hicks *et al.* 1974, Lewis 2000). However, water channels (aquaporins) and several other transport proteins have been identified in mammalian urothelium, and probably the alleged barrier is not always complete (Rubenwolf *et al.* 2009, Spector *et al.* 2002, Spector *et al.* 2011, 2013).

Currently, the accepted dogma is that urothelial permeability is consistent throughout the urinary tract. This is largely based on the assumption that morphologically the urothelium lining the ureter, bladder and urethra are the same. However, their embryological derivation is quite different, urothelium of the renal pelvis and the ureter being of mesodermal origin, while the lining of the bladder and urethra is of endodermal origin (Al-Kurdi 2017). There is also evidence suggesting that despite apparent histological homology, protein expression on the surface of urothelial umbrella cells is not consistent (Riedel *et al.* 2005), and Williams et al. (2016) showed differences in urothelial permeability between the upper urinary tract and the bladder.

Many factors, such as aquaporins and urea transporters, have been shown to be involved in the regulation of the urothelial lining, including its permeability normally and in different disorders (Kreft *et al.* 2009, Al-Kurdi 2017). For example, conditions such as partial bladder outlet obstruction, dehydration and urothelial carcinoma has been shown to affect aquaporin expression in the bladder (Rubenwolf *et al.* 2009, Spector *et al.* 2002, Kim *et al.* 2010, Rubenwolf *et al.* 2012). Previous studies on the American black bear indicated reabsorption of urine from the bladder, probably an advantageous ability during hibernation to prevent dehydration and improve hygiene (Nelson *et al.* 1973a, Nelson *et al.* 1973b, Spector *et al.* 2015). Most studies on changes in urine composition have focused on the storage period in the bladder, but although the transit time in the ureter is brief, the contact surface between urothelium and urine is considerable and a possible change might occur also in the ureter. Even though this has been addressed previously in human patients who had undergone treatment for urinary tract stones, or surgery including bilateral cutaneous ureterostomy (Cahill *et al.* 2003, Shafik *et al.* 2005), the issue has not been clarified. So, we want to further explore the possibility that the ureter has an important role in the regulation of urine composition under normal conditions. Since the pig is often used as a model in translational urological research, we employed a pig model to examine the changes of urine composition occurring when urine is passing from the renal pelvis to the bladder. In particular, we wanted to assess whether concentration changes primarily occur during the passage through the ureter, after entering the bladder, or during storage in the bladder. Such changes in concentration could be relevant when designing new treatments or diagnostic tests. The findings may have implications both regarding bladder dysfunction, but also in urinary stone disease.

Material and methods

Experimental animals and anaesthesia. Ethical approval of experiments was granted by the Danish Animal Experiments Inspectorate (Approval no: 2016-15-0201-00942). All procedures were carried out at Aarhus University and conformed to the Danish National Guidelines for care and handling of animals.

Female pigs (~40 kg) crossbred Landrace/Yorkshire/Duroc were obtained from a commercial source and kept at the farming facilities of Aarhus University at least one week prior to experiments, assuring health conditions. They were fasted over night with free access to water, and weighed at the day of the experiment. Anaesthesia was induced with intravenous injection of hypnomidate (0.5 mg/kg) and maintained with sevoflurane (~ 1.3 MAC) and intravenous infusion of fentanyl (12.5 µg/kg/h). Animals were intubated and ventilated automatically throughout the duration of the experiment, with continuous monitoring of temperature and vital parameters. All pigs were overhydrated following a standardised protocol during the experiment with intravenous infusion of equal volumes of isotonic sodium chloride and ringer acetate (in total ~1000 mL/hour), based on experience with low diuresis in anaesthetised pigs. Upon finishing the experiments animals were euthanized by intravenous injection of pentobarbital (40 mg/kg) while still anaesthetised.

Surgical procedures. Intravenous catheters were placed in superficial veins of both ears for anaesthesia and IV fluids. The left carotid artery was surgically exposed, and a vascular sheath was placed to monitor blood pressure and for drawing blood samples. A transurethral catheter (Rüsch, silicone, ch. 14, Teleflex Medical, Athlone, Ireland) was placed in the bladder. The renal pelvis on both

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sides was accessed retroperitoneally through small transverse flank incisions, and a pig-tail catheter (M-drain, 7 Fr, Mermaid medical, Copenhagen, Denmark) was inserted directly into the pelvis by Seldinger technique, and suture fixed. Afterwards a Pfannenstiel incision was made and the distal left ureter was exposed and cut at the entrance to the bladder. The bladder site was ligated. An 8 Fr baby-feeding tube (Unomedical, Lejre, Denmark) was inserted 2-3 cm into the distal left ureter and held in place with a suture.

Collecting and handling samples. The pig was placed in prone position and the four urinary catheters were each connected to a tap and placed in collecting tubes (Nunc Cryotube Vials, Thermo Scientific, Massachusetts, USA). Ten sets (forty samples) of simultaneously collected paired urine samples of 4 mL from the four collection sites were obtained from each pig. Experimental setting is depicted in figure 1.

Afterwards the bladder catheter was clamped and the bladder filled. If diuresis from the right side was insufficient, urine from the left side catheters was added. When reaching a bladder volume of 300 mL, confirmed by aspiration, the right ureter was ligated, leaving the bladder with a constant volume. Five additional samples of bladder urine were now collected with 30-minute intervals to evaluate concentration changes during urine storage in the bladder.

In order to exclude significant systemic changes during the experiment, a total of five blood samples from each pig were drawn from the central arterial catheter during the entire experiment, to evaluate serum concentrations of the examined parameters (Na^+, K^+, Cl^-, creatinine, urea and osmolality).

Each urine sample was immediately separated into 2 tubes and the pH was measured using an InLab®Semi-Micro electrode, connected to a SevenCompact™ pH-meter (Mettler Toledo, Hasselager, Denmark), automatically correcting for temperature. Hereafter both samples were snap frozen in liquid nitrogen and stored at -80° C until further analysis. Osmolality was determined using the OSMOMAT® 030 cryoscopic osmometer (Gonotec, Berlin, Germany). All other parameters were analysed in the

Advia$^{\circledR}$ 1800 (Siemens Healthineers, Erlangen, Germany) according to standard procedures. Creatinine and urea was measured by

spectrophotometry, with intra- and inter-assay precisions below 3 and 4 CV%, respectively. Sodium, potassium and chloride were

determined by ion selective electrodes, with intra- and inter-assay precisions below 2.7 and 3.7 CV%, respectively. After separation

blood samples were centrifuged at 3000 rpm for 10 min at 4° C. Serum was snap frozen and stored at -80° C until further analysis,

which was done following same procedure as with the urine samples.

Statistical Analysis – Statistical analysis was performed in Graphpad Prism 7.0a (GraphPad Software Inc., La Jolla, CA, USA). Results

from pH-measurements were converted into hydrogen-ion concentrations ($H^+=10^{-pH}$) for accurate statistical processing. The relative

concentration changes of all parameters were compared in pairs of upper sample (renal pelvis) and lower sample (bladder or distal

ureter) with a ratio paired t-test. The mean ratios were then combined for all pigs, and analysed by one-sample t-test against 1.

Assumptions for validity of testing were checked graphically with qq-plots, correlation scatter and Bland-Altman-plots. Linear

regression was performed to evaluate changes over time, analysing paired sample ratios, serum samples and the five bladder urine

samples from each pig, collected during urine storage at fixed volume.

Results

Six female pigs with a mean weight of 39.1 kg (range: 37.3-40 kg) were included in the study. Sixty sample sets were available to

compare renal pelvis and bladder urine. For comparison of renal pelvis to distal ureter urine, 48 sample sets were available, since

twelve sets had to be excluded due to technical issues such as haematuria or catheter malfunction (ten of the excluded samples were

from one pig). Thirty serum samples were available for osmolality, and 25 available for testing other parameters. The latter numbers

were the same regarding samples from prolonged storage in the bladder, where also 30 samples were available for pH measuring.

Urine alkalinity showed a marked and uniform increase on both sides, with a significant increase in pH from pelvis to bladder (mean decrease in H+: 32 % CI: 20-45 %) and from pelvis to distal ureter (mean H+ decrease: 23 % CI: 14-31 %), corresponding to a mean increase of 0.17 (0.10-0.26) pH points between pelvis and bladder, and 0.11 (0.07-0.16) pH points from pelvis to distal ureter (Fig. 2).

Concentration of all other parameters (Na^+, K^+, Cl^-, creatinine, urea and osmolality), showed a tendency to increase in the sample sets from the renal pelvis and the bladder. However, apart from the pH, these changes were statistically significant only regarding creatinine and K^+ (Table 1). Creatinine showed a mean increase of 17 % (CI: 2-32%) and K^+ a mean increase of 12 % (CI: 1-23 %). Evaluating the sample sets comparing the renal pelvis and the distal ureter did not show consistent results on any parameters, apart from the pH. We did not observe any other changes that were statistically significant in these sample sets (Table 1).

The direction of concentration changes regarding all parameters was not uniform, but showed some sample pairs with increasing concentrations and some with decreases, as illustrated in figure 3a+b. Generally, a low concentration in the pelvis would warrant a prominent increase in concentration, whereas a high concentration in the pelvis would decrease or remain unchanged in the lower sample (fig 3a+b). In some instances, this tendency was extremely marked, as illustrated in figure 3c, and this effect gives rise to the tendency of urea to decrease through the ureter, a finding mainly based on sample sets with a high concentration in the pelvis.

The final part of the experiment, evaluating concentration changes during storage in the bladder with a fixed volume, showed increasing concentration of urea in all pigs (Figure 4). Regarding all other parameters (Na^+, K^+, Cl^-, pH, creatinine and osmolality) results were diverging between pigs, with some pigs showing decreases and other pigs increases in concentration during storage. Considering all pigs together urea concentration showed a mean increase of 14% (CI: 1-27%), all other parameters showed no significant changes.

Relative concentration changes between samples from the renal pelvis and samples from the bladder or distal ureter were overall not affected by duration of anaesthesia as evaluated graphically and by linear regression (data not shown).

Serum concentrations of the examined parameters were overall stable throughout the experiment regarding most parameters. There was, however, an overall mean decrease of 2.3 % (CI: 0.7-4.0 %) per hour in serum creatinine as estimated by linear regression (p=0.02).

Discussion

We found an increased urine alkalinity during passage of the ureter and a slightly larger increase after entry of urine into the bladder. However, no further significant changes in pH occurred after prolonged storage of urine in the bladder. This could indicate that the reduction in H^+ concentration is predominantly occurring in the ureter. Concentrations of creatinine and K^+ increased significantly from the renal pelvis to the bladder. During prolonged storage in the bladder we observed an increasing urea concentration.

Our findings are consistent with those shown in human subjects by Cahill *et al.* (2003). Their studies were performed on mainly unilateral samples from patients with renal stones, comparing concentrations in the operated renal pelvis to concentrations in bladder samples. They further had a few sample sets from both renal pelvises, and found no significant differences between the two sides. In addition, they found higher pH, Na^+, K^+ and osmolality in bladder samples compared to renal pelvis samples, and furthermore observed (the same effect as we did) that the direction and degree of concentration changes depends on the primary concentration. However, they were not able to distinguish between changes occurring in the ureter and those occurring in the bladder.

With our animal model design, we can speculate that since the pH increased significantly in the sample pairs comparing distal ureter to the renal pelvis, at least part of the change must occur in the ureter. Shafik and colleagues (Shafik *et al.* 2005) conducted a series of studies, all examining urine sample pairs from human subjects. In one study, they examined concentration changes occurring in the urethra by sampling voided and vesical urine. Surprisingly, they found increases in both pH, Na^+, K^+ and osmolality, which they attributed to either the interaction between urine and urothelium in the urethra or alternatively to the addition of mucus from

prostatic and urethral glands (Shafik *et al.* 2004). In another study Shafik *et al.* (2006) compared samples from the right renal pelvis to samples from the bladder, disregarding the contribution from the left kidney. They observed increases in all examined parameters; pH, osmolality, Na^+ and K^+. In a further study they focused on changes occurring through the passage of the ureter (Shafik *et al.* 2005), and compared urine samples from the renal pelvis with samples from uretero-cutaneostomies in patients who had previously undergone cystectomy. They concluded that concentrations of Na^+ and K^+ increase, and that only insignificant increases in pH and osmolality occurred. Contributing to the differences in results between our study and that of Shafik et al. could be that urinary pH is different and that they studied patients, whereas we studied normal pigs. Their human subjects had a urinary pH~6 as compared to our pig subjects with pH~7.3. We demonstrated a mean decrease in H^+ concentration of 23 % through the ureter. The larger change we observed in the sample sets, where the urine had passed the ureter as well as the bladder (32 %) suggest that there is a further change occurring in the bladder. However, the results after prolonged storage in the bladder where urine alkalinity does not change significantly contrarily indicate that the alkalinisation primarily happens in the ureter. The transit time through the ureter is short, but the contact surface between urine and urothelium is much larger than in the bladder, justifying the assumption of changes occurring predominantly in the ureter. As mentioned in the introduction, there may be differences in permeability between the urothelium in the upper tract compared to that in the bladder. However, the baseline pH of the bladder urine was significantly higher than pH of renal pelvis urine, and this might also explain the lack of significant change during prolonged storage. These pH changes might be especially relevant in patients suffering from urinary stone disease, as pH influences the likeliness of stone formation. It is relevant to know that a urine sample from the bladder might not reflect the pH found in the pelvis, where the stones are usually formed.

Thus, the findings in our present study are in concordance with previous studies regarding the direction of concentration changes, but we find diverging answers to the question whether the pH is mainly affected by the passage of the ureter or by the storage in the bladder. Rubenwolf *et al.* (2012) developed a cultured differentiated cell layer of human urothelial cells and demonstrated permeability to radioactively-labelled water and urea, with an increased flux after establishing an osmotic gradient. They further demonstrated expression of AQP3, 4, 7 and 9, and found upregulation of AQP3 after exposure to hyper-osmotic NaCl and

downregulation caused by hypo-osmotic NaCl. AQPs were then blocked with $HgCl_2$ and permeability re-evaluated. Permeability to both water and urea was reduced following AQP-blockage by $HgCl_2$ in a concentration dependent manner, indicating that the permeability might be mediated by AQPs. In a previous study, also by Rubenwolf *et al.* (2009), expression of AQP3, 4, 7, 9, and 11 transcripts was demonstrated in samples from human ureter. In a recent study we demonstrated expression of AQP1, 3, 9, and 11 transcripts in the foetal porcine ureter (Jakobsen *et al.* 2018). The increases in concentration of all evaluated parameters (except for H+, which decreases) would correspond well with active reabsorption of water from the urinary tract. The regulation of urinary acidification has been well studied in the kidney, and the main processes involved in the maintenance of systemic acid-base homeostasis are reabsorption of filtered HCO3 and excretion of acid and ammonium. A variety of acid-base transporters are involved in the processes of urinary pH regulation. The collecting ducts are considered the final site for urine acid-base regulation, but our findings suggest that the regulation continues at least through the ureter. Embryologically, both the ureters and the collecting ducts originate from the ureteric bud. With that in mind, similarities in their function seems plausible. A urinary acidification defect caused by diminished net H+ secretion and/or HCO3-reabsorption has been shown following urinary tract obstruction (Wang *et al.* 2008). However, obstruction also affects the ureters, and it cannot be excluded that some of these transporters are located also in the urothelium of the ureters and play a role in the changes in H+ concentration occurring in the normal situation. Obviously, this possibility could motivate further study. To increase diuresis in our model, all pigs were overhydrated with intravenous infusion of equal volumes of isotonic sodium chloride and ringer acetate during the experiment. Spector *et al.* (2011, 2013) studied how urothelial transport of water and solutes may occur and may be regulated physiologically by animal hydration status. They instilled urine collected during 2-day water deprivation, water loading, or ad libitum water intake into isolated in situ bladder(s) of groups of rats undergoing one of the same three hydration states. They retrieved the urine and measured changes in urine volume and concentrations and quantities of urine urea nitrogen, creatinine, and other solutes. For each ionic species, two factors independently influenced transport: Instilled urinary ion concentration and animal hydration state. To what extent the hydration state may have influenced our results can only be speculated. Since all pigs were subjected to the same degree of hydration, qualitative differences would not be expected. On the other hand, compared to normal, quantitative differences cannot be excluded.

In conclusion, this study has shown that changes in urine composition occur when urine passes from the renal pelvis to the bladder and during storage in the bladder. Our results suggest that some of the changes occur already during the passage through the ureter. The transport mechanisms involved and the implications of the findings motivate further study.

Acknowledgements

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	Relative changes			
	Pelvis to bladder	p	Pelvis to distal ureter	p
Osmolality	1.06 (1.00-1.11)	0.06	1.01 (0.91-1.11)	0.81
K+	1.12 (1.01-1.23)	0.04	0.96 (0.73-1.19)	0.65
Na+	1.03 (0.98-1.07)	0.16	1.03 (0.97-1.10)	0.25
Cl-	1.04 (0.98-1.09)	0.14	1.02 (0.94-1.09)	0.60
Creatinine	1.17 (1.02-1.32)	0.03	0.97 (0.72-1.21)	0.71
Urea	1.15 (0.96-1.33)	0.09	0.87 (0.70-1.04)	0.10
H+ (10^{-pH})	0.68 (0.55-0.80)	<0.01	0.77 (0.69-0.86)	<0.01

Table 1

Mean relative changes in concentration (95% confidence intervals)
Compared as Lower/Upper ratio – Lower= Bladder or distal ureter, Upper= Renal pelvis
Significant changes are marked by coloured background.
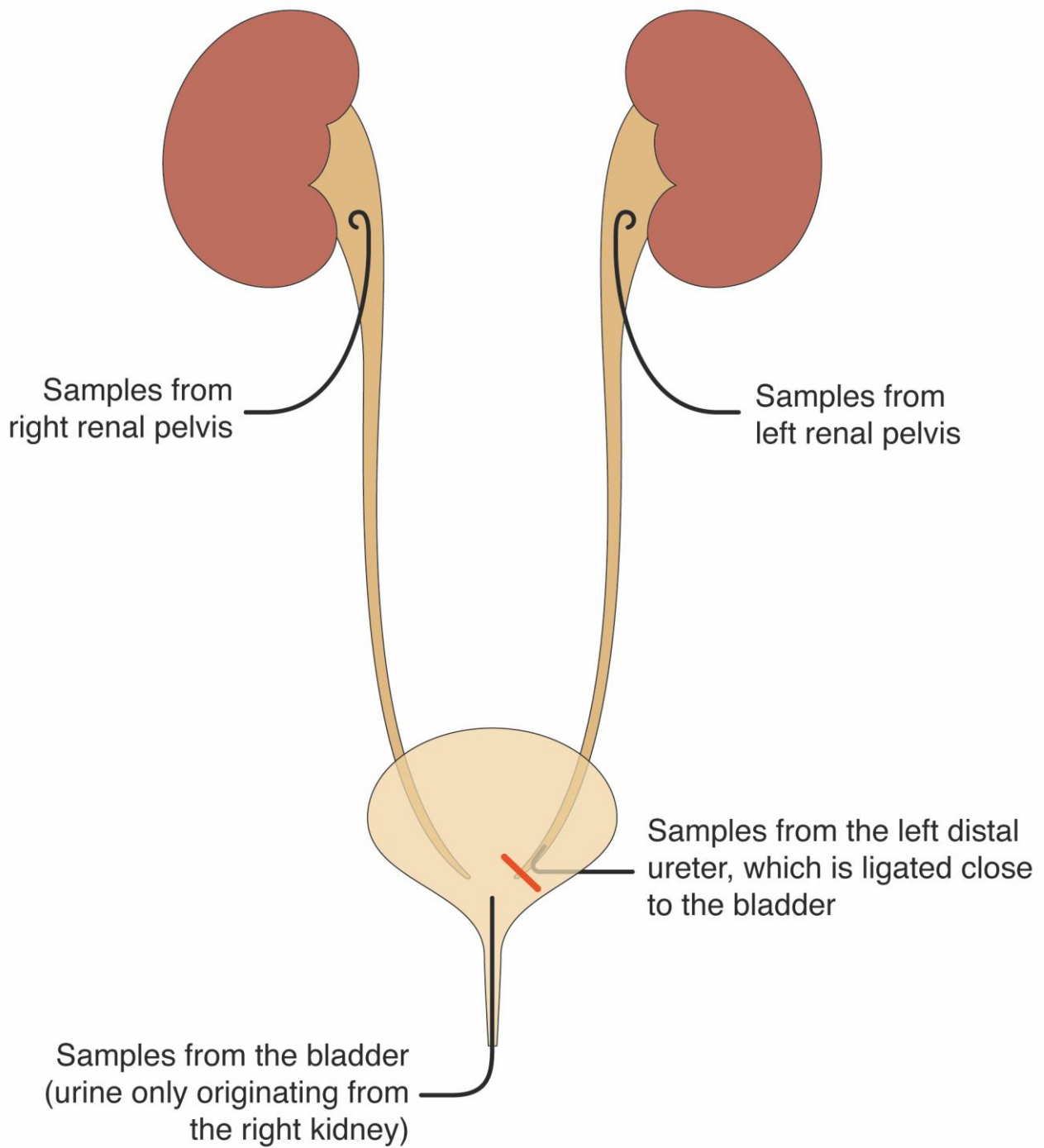p-values <0.05 reject the hypothesis of ratio=1.

Figure 1 – Experimental setting

To evaluate changes from the renal pelvis to the bladder, samples are taken from these locations on the right side.
On the left side, samples are taken from the renal pelvis to the distal ureter, and the entry point of the left ureter to the bladder is closed, to avoid interference with the right side sample sets.
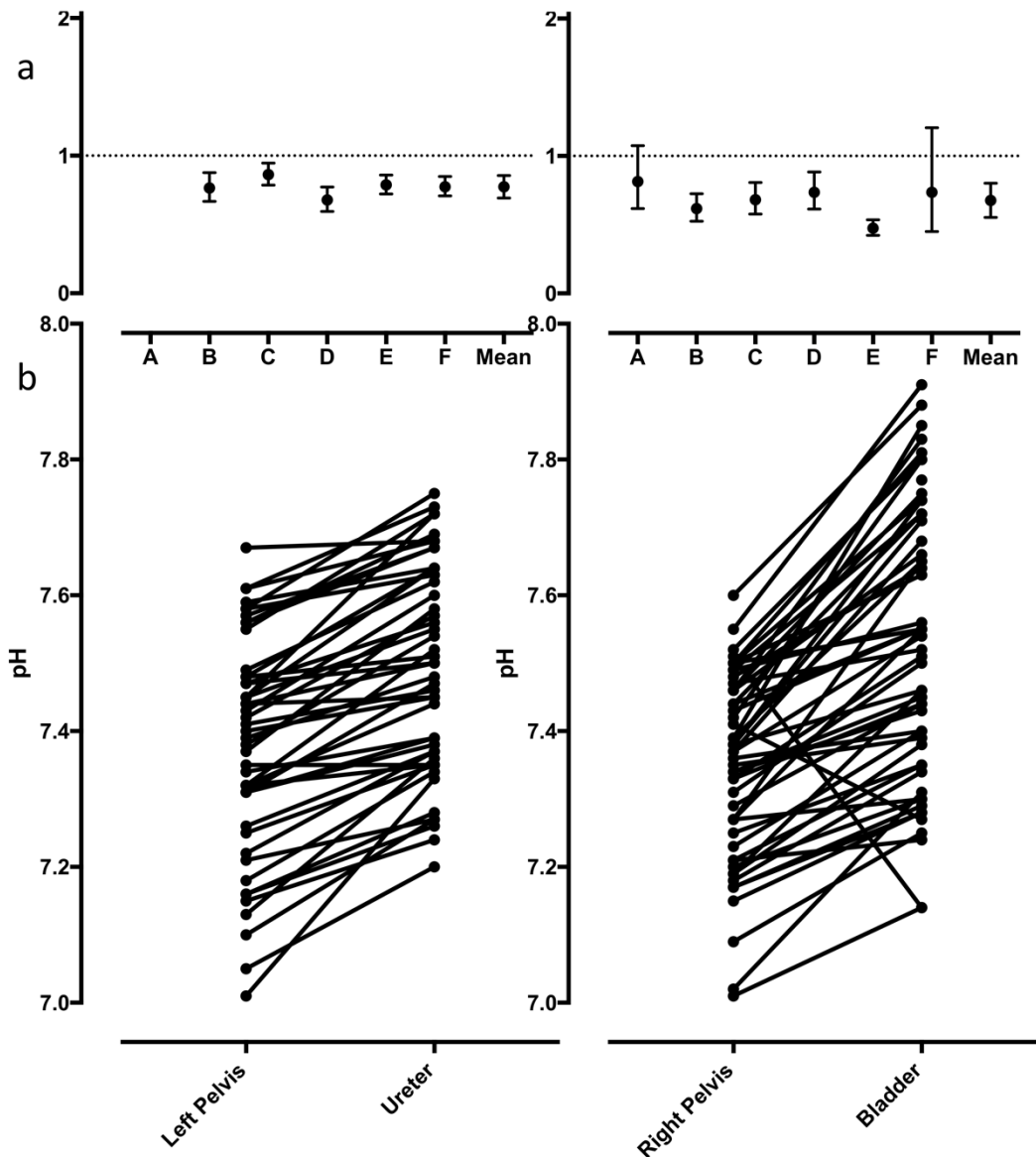
Figure 2 - Changes in pH from renal pelvis to either distal ureter or bladder

a) Mean relative changes in separate pigs (A-F) and the overall mean relative change (Mean).
Samples comparing renal pelvis to distal ureter on the left, samples comparing renal pelvis to the bladder on the right.
(Error bars: 95% confidence intervals, dotted line: no change)

$$y = \frac{H^+ concentration\ lower\ sample\ (bladder\ or\ distal\ ureter)}{H^+ concentration\ renal\ pelvis}$$

b) Plots of pH changes in paired samples from both sides. Sample location indicated on the x-axis. pH increases in most sample pairs.
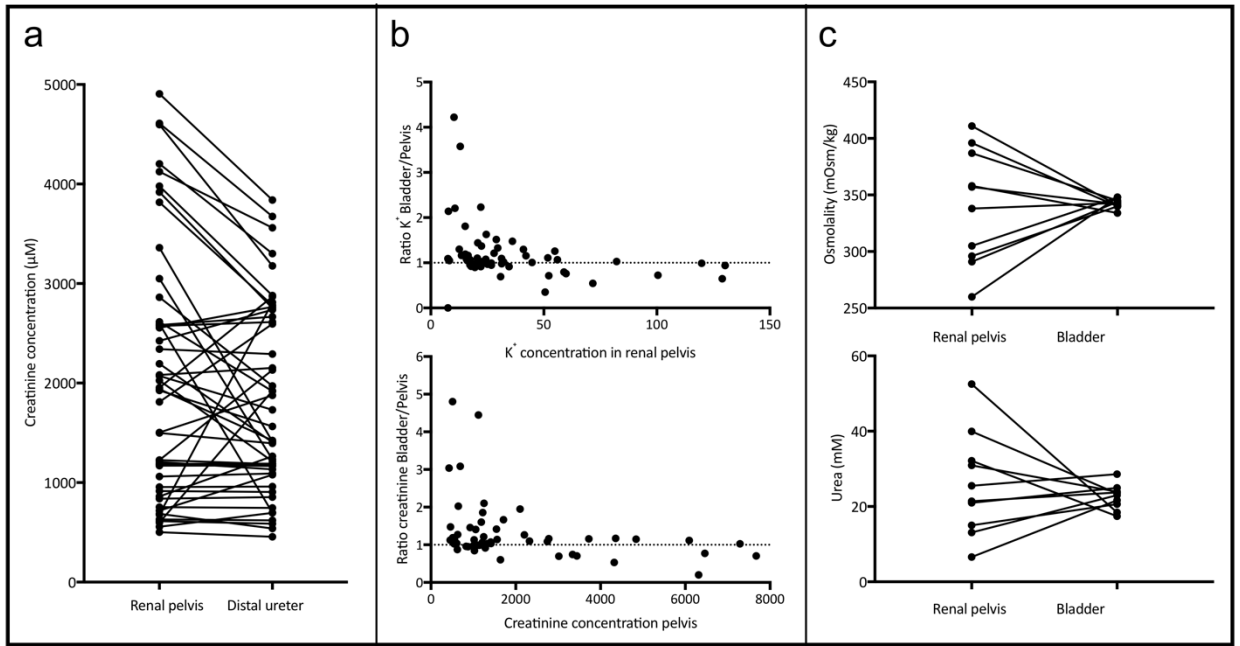
Figure 3

a) Plots of creatinine concentration in paired samples evaluating changes from the renal pelvis to the distal ureter. Direction of changes is not uniform between sample sets. In general, when starting at a high concentration there will be a decrease towards the lower sample, and vice versa, as further illustrated in panel b.

b) The relative changes in concentration of K^+ (top) and creatinine (bottom) plotted as a function of the concentration in the renal pelvis. Relative increases are greater when the renal pelvis concentration is low.

c) Changes of osmolality and urea concentration from one pig showing a marked example of the above described effect.
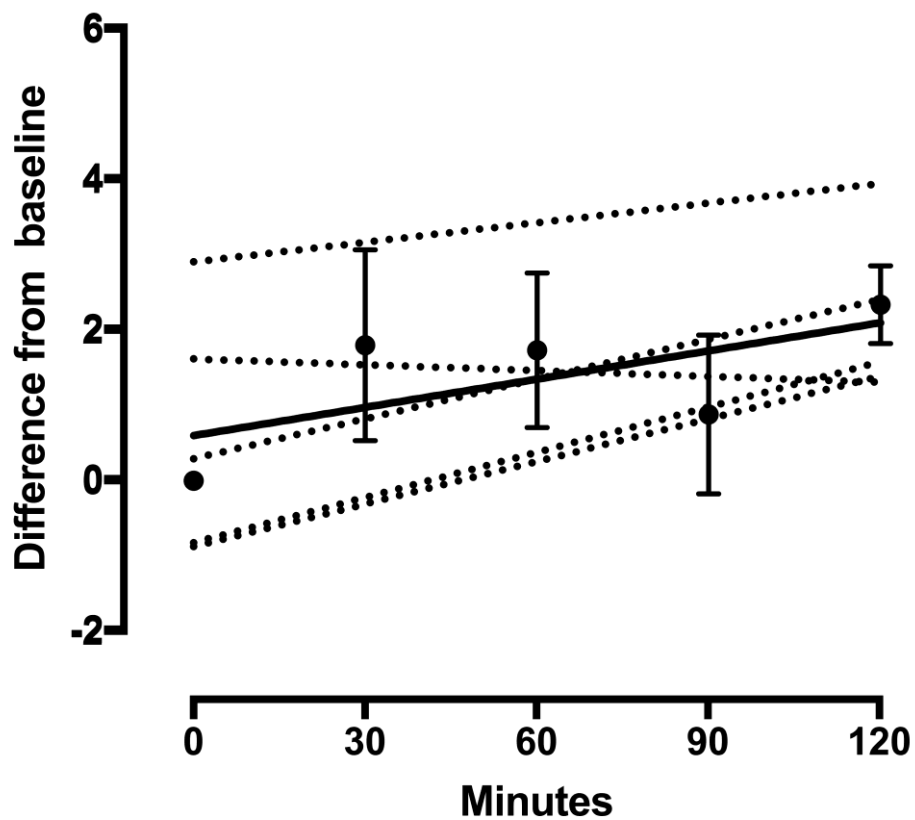
Figure 4

Change of urea concentration during two hours of storage in the bladder. On the y-axis is indicated the absolute change from baseline concentration. The dotted lines represent fitted linear regression curves from individual pigs. The solid line is the overall curve-fit. Dots and error-bars represent mean values and SEM.