Renal Contributions to Water Balance in the Anna's Hummingbird, *Calypte anna*.

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Abstract - 1. Glomerular filtration rate and fractional water reabsorption were measured in six Anna's hummingbirds under different water intake rates.

2. Glomerular filtration rate was not higher than predicted by body mass. Large water loads do not appear to have selected for high filtration rates.

3. Fractional water reabsorption was high enough that excretion rates were always less than intake rates. The difference between water intake rate and excretion rate significantly increased ($p < .0005$) as water intake rate increased.

4. Under large water loads, evaporative water losses may play a more significant role in water balance than previously predicted.

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Introduction

The hummingbird has one of the highest mass-specific metabolic rates of any vertebrate (Pearson, 1950; Laswieski, 1963; Bartholomew and Lighton, 1986; Powers and Nagy, 1988; Powers, 1992), which requires that they balance energy requirements on a daily basis (Hixon, 1982). This large energy demand is complicated by the fact that hummingbirds are nectarivorous; they are forced to consume large quantities of water. A free-ranging 4.5 g Anna's Hummingbird turns over 164% of its body mass in water per day (Powers and Nagy, 1988). This value is 89% greater than expected for a bird of this size (Nagy and Peterson, 1988), and is one of the highest values for any vertebrate measured (Powers and Nagy, 1988). Excreted fluid osmolalities are generally less than 100 mOsm/kg, which is only one-forth to one-sixth the osmolality of typical avian plasma (Calder and Hiebert, 1983). This very dilute excrement demonstrates the hummingbird's need to eliminate water. Powers and Nagy (1988) found that for a 4.5 g hummingbird at 24° C, water balance could be maintained by a water turnover rate of 7.35 ml/day. Subtracting evaporative water loss (EWL) (Laswieski, 1964; Powers, 1992) leaves a net of about 5 ml to be excreted by the kidney per day (Beuchat *et al*, 1991; Powers, 1992). Other estimates have predicted similar renal water loads (Calder, 1979). Because 5 ml is such a large volume to be excreted by a 4.5 g hummingbird each day (111% of body mass), it is possible that the hummingbird kidney has become specialized to handle these incredible water loads.

The kidneys of two hummingbird species, *Selasphorus platycercus* and *S. rufus*, were examined by Johnson and Mugaas (1970). These kidneys were found to "contain a poorly developed renal medulla consisting almost entirely of collecting ducts with only a few associated looped nephrons" (Johnson and Mugaas, 1970). A single kidney from a black-chinned hummingbird, *Archilochus alexandri*, apparently lacked medullary cones (Beuchat *et al*, 1991; Beuchat pers comm). Since it is the looped nephrons of the medullary cones which are responsible for concentrating urine (Braun

and Dantzler, 1972), the limited number of these nephrons demonstrate specialization of the hummingbird kidney which is consistent with the need to produce a dilute urine. Concomitant specialization of physiological processes might be expected of the hummingbird kidney.

Glomerular filtration rate (GFR) "is the primary, if not always the sole, determinant of the rate at which fluid is delivered to the corresponding proximal tubule and therefore is the initial determinant of the volume and composition of the final urine" (Dantzler, 1988). Because of this important role in regulating renal function, GFR is a physiological parameter which one might expect to have become specialized in hummingbirds. However, GFR is not the only determinant in the ability of the hummingbird to remove excess water. Fractional water reabsorption (FWR) must also be considered.

The general pattern of filtration followed by reabsorption is the pattern of renal homeostasis that has been favored by evolution (Beyenbach, 1985). Glomerular filtration and tubular fluid reabsorption are processes which are functionally linked as the mechanism of extracellular fluid turnover (Beyenbach, 1985). Therefore, any specialization in the magnitude of GFR will affect tubular reabsorption. For example, an increase in GFR requires a proportionate increase in the reabsorption of needed solutes and water if these are not to be voided as excrement.

The most recent allometric relationships predict that a 4.5 g hummingbird has a glomerular filtration rate of about 3.5 ml/h (Yokota *et al*, 1985; Williams et al, 1991). At this filtration rate, a hummingbird could eliminate its water load renally with a fractional water reabsorption (FWR) of about 66-88% (Beuchat *et al*, 1991). However, these FWR values are "substantially lower than the FWR typical of terrestrial birds and mammals" (Beuchat *et al*, 1991). Alternatively, a hummingbird with a FWR of 95% would require a GFR of 8.4 - 23.8 ml/h in order to eliminate the same water

load renally. Either scenario involves specialization of the processes involved in renal water elimination.

Extra-renal water losses could bypass the need for enhanced renal function, and potentially allow an energetic savings. It is likely that EWL in hummingbirds may be considerably higher in the field than under the laboratory conditions used by Laswieski (1964) and Powers (1992). EWL in flying birds is several times higher than in resting birds (Hart and Roy 1966; Tucker, 1968; Berger *et al*, 1971; Dawson, 1982). If evaporative losses constitute a major avenue of water loss, then the hummingbird kidney would not need to excrete the large renal water loads which have been predicted (Calder, 1979; Beuchat *et al*, 1991; Powers, 1992).

The purpose of this study was to examine the role of the kidney in water balance in hummingbirds. Glomerular filtration rate and fractional water reabsorption were measured in restrained, conscious Anna's hummingbirds, *Calypte anna*. Water intake during measurement periods was experimentally adjusted between measurements in order to examine the influence of water load on physiological function.

Materials and Methods

Six Anna's hummingbirds were captured in suburban San Diego in the summer of 1991. These individuals were housed in wire cages (approx. 0.5 m^3) at San Diego State University and were under a natural light cycle. Birds had access to Nekktar Plus brand (Nekton Corp.) hummingbird food *ad libitum*. All birds appeared to be active and healthy throughout the study. The average mass during the study was 5.1 ± 0.9 g.

^{3}H-polyethelyene glycol (^{3}H-PEG, MW = 4000) was used as a filtration marker for measurement of GFR. About 10 ul of ^{3}H-PEG solution (150 uCi/ml) was introduced into the birds by subcutaneous injection. GFR was calculated as $U_{PEG} \cdot V_u / P_{PEG}$, where U_{PEG} is the concentration of ^{3}H-PEG in the urine (dpm/ml), V_u is the urine flow rate (ml/h), and P_{PEG} is the plasma concentration of ^{3}H-PEG (dpm/ml). The

excretion rate of ^{3}H-PEG ($U_{PEG} \cdot V_u$) was measured by collecting spontaneously voided excrement samples from a plastic pan placed beneath the birds. The samples were drawn into microcentrifuge tubes by light vacuum. Microcentrifuge tubes were then sealed to prevent evaporation.

Hummingbirds were restrained in a paper jacket throughout measurements. A small window was made in the jacket over the back of the birds for introduction of the ^{3}H-PEG solution. The introduced solution remained visible as a small reservoir beneath the skin of the back throughout the measurement period. Birds were fed a simulated nectar solution (.6M sucrose) during the experiment and the volume of solution consumed was measured.

Plasma samples (9 ± 6 ul) were taken by clipping a toenail and collecting the blood in heparnized capillary tubes. Samples were immediately centrifuged to separate plasma from red blood cells. Plasma volume and ^{3}H-PEG activity were then measured. Plasma samples were taken at the start and end of clearance periods, except for one measurement period in which a single sample was taken toward the middle of the clearance period.

Clearance periods lasted for an average of 70 minutes. The amount of ^{3}H-PEG excreted during the clearance period was grouped into ten minute time intervals within each clearance period. Least squares linear regression was used to determine ^{3}H-PEG excretion rate from these collection intervals.

FWR was calculated as GFR - V_{H_2O}/ GFR, where V_{H_2O} is the excretion rate of water throughout the experiment. This excretion rate was measured by weighing the excreted samples on a Mettler top-loading balance at the end of a measurement period. The assumption was made that all excreta was water that came from the kidneys. Water reabsorption which could potentially occur in the cloaca will cause overestimation of renal FWR. Excrement passing through the GI tract will cause underestimation of renal FWR. Water intake rate and excretion rate were measured from the time that birds were restrained

to the time that they were put back into the cage. These time periods overlapped and included clearance periods.

^{3}H-PEG from plasma and excrement samples was assayed using liquid scintillation counting procedures. Results are reported as means ± 1SD. Linear regression and statistical analyses were done on a Macintosh using Statview SE + Graphics. Regressions were considered statistically significant if $p \leq 0.05$.

Results

The mean GFR for the 12 measurement periods was 2.40 ± 0.8 ml/h . This value is only about 60% of the 3.9 ml/h predicted for a 5.1 g bird (Williams *et al*, 1991; Yokota *et al*, 1985). GFR was not correlated with body mass, water intake rate, or FWR.

Measurement of GFR in hummingbirds offers special challenges which are not encountered with measurements in larger birds. Some of these challenges are a result of the simple fact that small size restricts the choice of measurement techniques. For example, intraperitoneal implantation of osmotic minipumps in GFR measurements is an option for use in starlings (Dantzler and Roberts, 1989) and Gambel's quail (Williams *et al*, 1991), and even in song sparrows (Rothschild and Goldstein, 1990). However, the size of the minipumps is too large for use in a 5 g hummingbird (Beuchat pers comm).

Similarly, measurement of plasma ^{3}H-PEG concentration is more difficult in hummingbirds than larger birds because of the small absolute plasma volume available for sampling. A 5 g hummingbird has only about 200 ul of plasma, which necessitates small plasma samples. Plasma volumes in this study were measured in 1-5 ul aliquots. The variability of these aliquots as determined from 63 pipetting trials was 9.7% of the mean. This variability is certainly higher than larger aliquots would yield.

Physiological parameters associated with small body mass can potentially cause special problems for GFR measurement. Small birds filter a higher percentage of their total

plasma volume per unit time than larger birds. This fact is arises because GFR (ml/h) in birds scales with body mass (g) as 1.29 $M^{0.68}$ (Williams *et al*, 1991), while plasma volume scales with body mass (g) as about .04 $M^{1.0}$ (Calder, 1984). This means that the percentage of total plasma filtered per minute scales with body mass (g) as 53.7$M^{-0.32}$. Based on this relationship a 4.5 g bird filters 33 %, a 25 g bird filters 19 %, a 100 g bird filters 12 %, and a 34 kg emu only filters 2 % of total plasma volume per minute. Therefore, any changes which alter plasma ^{3}H-PEG concentration or ^{3}H-PEG excretion rate during a clearance period occur more quickly in a small bird than in a large bird.

This more sudden change in small birds has important implications for GFR measurements. During any clearance measurement, it is assumed that the measured plasma marker concentration is representative of the concentration during the clearance period. However, the plasma measurement is representative of a concentration at a discrete point in time, while the excretion rate for a clearance period is determined over a time continuum. Measurement errors will occur if the plasma marker concentration is not representative of the concentration throughout the clearance period. This type of change is possible in any GFR measurement, but is more probable in small birds because they filter a higher percentage of their total plasma volume per unit time than larger birds.

Mis-matching of excretion rate and plasma concentration will occur if GFR changes within a clearance period. Alteration of GFR is an important point of osmotic regulation in birds (Braun, 1982). Dehydrated birds exhibit significantly lower GFR's than do hydrated birds (Williams *et al*, 1991). This response is apparently mediated by the neurohypophysial peptide hormone, arginine vasotocin (AVT) (Braun, 1982; Stallone and Braun, 1985; Gerstberger *et al*, 1985). Under experimental conditions, GFR alterations can occur rapidly. Following injection of synthetic AVT (10 ng · kg^{-1}), GFR in the duck significantly decreased within 10 minutes (Gerstberger *et al*, 1985). An acute salt load in the Starling significantly increased GFR within 40 minutes (Laverty and Wideman, 1989).

The fact that small animals have faster physiological time scales (Calder, 1984) means that they must respond to physiological changes more quickly than larger animals in order to maintain homeostasis. Because GFR is an important point for regulating kidney function, it should be important for small birds to have the capacity to alter GFR even more quickly than large birds. Dramatic changes of excretion rate in at least one measurement, which was not used for GFR determination, suggests that hummingbird GFR can markedly change in a short time period (Fig. 1). The almost non-existent excretion rate during part of this measurement is consistent with virtual GFR cessation, as hypothesized by Calder and Hiebert (1983) and Beuchat *et al* (1991). Changes in GFR during a clearance period represent a potential problem for hummingbird GFR measurements.

In spite of potential problems, the variability in ^{3}H-PEG excretion rate and plasma ^{3}H-PEG concentration present during the 12 clearance periods in this study was in the range of expected measurement variability. Because excreted fluid is stored in the cloaca before it is voided, some resolution of the excretion rate may be lost. Artifacts caused by cloacal storage are more of a problem during short time periods, because ultimately, all cloacal ^{3}H-PEG will be excreted. Resolution of excretion rate greatly increased after grouping samples into 10 minute time intervals. Variability about the excretion rate during all twelve measurements was minimal ($R^2 = 0.994 \pm 0.003$).

The average difference between mean plasma ^{3}H-PEG concentration and high or low values within a clearance period was 9.3 ± 5.7 % of the mean, which is consistent with the variability (SD = 9.7 % of the mean) found from the 63 aliquots measured in the pipetting trials. There was no correlation between plasma ^{3}H-PEG concentration within a clearance period and the time at which a sample was taken, which indicates that plasma ^{3}H-PEG had reached equilibrium.

The mean FWR for 11 measurement periods was 93 ± 3.5 %. FWR significantly decreased ($p < 0.005$) as water intake rate increased. However, FWR was always higher than needed to match water excretion rate with water intake rate.

Water intake rates ranged from 0.13 to 0.78 ml/h. Water excretion ranged from 0.05 to 0.31 ml/h , but excretion rate was always lower than intake rate. The difference between intake rate and excretion rate increased significantly ($p < 0.0005$) as water intake rate increased.

Discussion

In birds, it is not clear that the general need to either conserve or eliminate water influences GFR (Yokota *et al*, 1985). Even though certain xeric species have lower GFR's than more mesic species (Yokota *et al*, 1985), these low rates may be associated with physiological parameters not directly linked to the need to limit renal water excretion. Williams *et al* (1991) suggest that the unexpectedly low GFR of the Gambel's quail, *Callipepla gambelii*, may be a result of their unusually low resting metabolic rate (Goldstein and Nagy, 1985). The most evident trend in GFR is that it is correlated with mass specific metabolic rate (Yokota *et al*, 1985). GFR scales with the same mass exponent as metabolic rate, and mammals and birds have significantly higher GFR's than do the ectothermic vertebrates (Yokota *et al*, 1985).

Using the data from the Anna's hummingbird and 20 other avian species, a new equation relating GFR to body mass is derived here (Fig. 2). The mean hummingbird GFR of 2.4 ml/h is below this line, and lower than predicted by other allometric equations (Calder and Braun, 1984; Roberts *et al*, 1985; Yokota *et al*, 1985; Williams *et al*, 1991). Incidentally, the kidneys examined by Johnson (1968) from two hummingbird species, *Selasphorus platycercus* and *S. rufus*, were lower in mass than predicted from an allometric analysis including 179 other avian species. The fact that hummingbird GFR is not higher than expected by body mass, even though the hummingbird has an extremely high water turnover rate, supports the idea that avian GFR is not specialized to meet the environmental factors which influence water load.

The intense mass-specific metabolism of the hummingbird may limit the amount of energy devoted to renal water elimination. The endothermic kidney already receives a large allocation of blood flow, despite its relatively small size. The avian kidney mass is only 1% of total body mass (Johnson, 1968), yet if comparable to mammals, receives about 26% of cardiac output (Edwards, 1975). The mass-specific metabolism of kidney tissue is 17 times higher than the average for the whole body (Calder, 1984). Because the high mass-specific metabolic rate associated with small size means that smaller animals are on a less "flexible" energy budget than larger animals, it would be especially surprising to find additional renal energy allocation in a 5 g animal.

The birds in this study excreted only a fraction of the ingested water load. Although FWR significantly decreased ($p < 0.005$) as water load increased, the birds always reabsorbed more water than necessary to match water excretion rate with water intake rate. At any given GFR, the difference between measured FWR and the FWR required to match water excretion rate with water input rate input rate significantly increased ($p < 0.01$) as water intake rate increased (Fig. 3).

Because the hummingbirds in this study reabsorbed such a large fraction of the filtered load, water intake rates were always higher than water excretion rates. The water excretion rate was only 45 ± 18 % of the water intake rate . This ratio was not significantly correlated with intake rate ($p > 0.05$). However, the absolute difference between water intake rate and water excretion rate significantly increased ($p < 0.0005$) as intake rate increased (Fig. 4). This relationship predicts that at a water intake rate of 7.2 ml/day at a mean ambient temperature of 24° C (Powers and Nagy, 1988), a hummingbird would take in 4.5 ml/day more than would be excreted renally.

A similar situation is seen in two other nectarivorous avian species. Two species of Australian honeyeaters, *Lichmera indistincta* and *Acanthorhynchus superciliosis*, took in more water than they excreted cloacally (Collins, 1981). Combined data from these species shows that, as in the hummingbird, the absolute difference between nectar intake rate and

cloacal excretion rate significantly increased ($p < 0.005$) as nectar intake rate increased (Fig. 5).

In another study, Gambel's quail excreted only 79% of an infused water load (Braun and Dantzler, 1975). The authors submitted that the isosmotic reabsorption of sodium and water in the proximal tubule may have prevented significant volumes of filtrate from reaching the diluting segments of the nephron, thus limiting total water output. This hypothesis offers a plausible explanation for why birds might be limited in the amount of water that they are able to eliminate. A 5 g hummingbird with a GFR of 2.4 ml/hr, filters its entire plasma volume every five minutes. This means that every five minutes it must reabsorb its entire plasma supply of glucose, electrolytes, amino acids, and other needed solutes. "Under normal physiological conditions the reabsorptive transport of Na, HCO_3, Cl, glucose, and amino acids by glomerular proximal tubules leads to net reabsorption of water in nearly isosmotic proportions" (Beyenbach, 1985). This link between solute and water reabsorption in the proximal tubule may make high FWR inevitable. Therefore, hummingbirds may be more dependent on extra-renal routes of water loss than predicted from EWL studies.

EWL has been measured in hummingbirds in two independent studies (Laswieski, 1964; Powers, 1992). The highest rate measured by Laswieski was 33.6 mg $H_2O \cdot (g \cdot h)^{-1}$. This was from a Costa's hummingbird, *C. costae*, which "showed signs of what appeared to be heat prostration"(Laswieski, 1964). The highest rate measured by Powers was 37.1 mg $H_2O \cdot (g \cdot h)^{-1}$. At 37.1 mg $H_2O \cdot (g \cdot h)^{-1}$, a 4.5 g hummingbird could lose 2.0 ml of water over a 12 hr day. These values predict that only about 27% of the predicted 7.35 ml taken in during a day (Powers and Nagy, 1988) would be lost through EWL.

However, the patterns of evaporative water loss during these laboratory measurements are probably not representative of typical patterns in the field. The birds in

both studies were resting in the dark during EWL measurements . The metabolic rate of hummingbirds in the thermal neutral zone (33-37°C) in Powers' study was about 35 $W \cdot kg^{-1}$ (Powers, 1992). This rate is only 39% of the field metabolic rate measured by Powers and Nagy (1988). In addition, birds were fasted (thus, water deprived) for 2 hours before measurements began, and these measurements lasted for 2-3 hours (Laswieski, 1964; Powers 1992). Activity level and hydration state both dramatically influence patterns of EWL in birds.

Hydration state has a significant effect on EWL in birds. EWL is significantly higher in more fully hydrated honeyeaters (Collins, 1981), pigeons (Webster *et al*, 1985), zebra finches (Calder, 1964; Lee and Schmidt-Nielsen, 1971), and budgerigars (Greenwald *et al*, 1967) than in water deprived birds. In the zebra finch (Lee and Schmidt-Nielsen, 1971), the difference in EWL was a result of a difference in rate of cutaneous water loss between hydrated and water deprived birds. Cutaneous water losses generally account for about 50% of the total water loss in birds even though they do not possess sweat glands (Dawson, 1982). Webster *et al* (1985) suggested that changes in cutaneous evaporative water loss could be caused by conformational changes in lipids in the epidermis, or to changes in blood flow/hydration state of the keratin layer.

Flight in birds also has a dramatic influence on evaporation. EWL has been measured during flight in the budgerigar (Tucker, 1968), the pigeon (Hart and Roy, 1966), and the black duck (Berger *et al*, 1971). In all cases, EWL during flight was several times higher than during resting states. At 35° C, the budgerigar has a flying evaporation rate 12 times higher than when resting (Tucker, 1968). The pigeon's evaporation rate in flight is up to 20 times higher than when resting (Hart and Roy, 1966), and the black duck evaporates respiratory water 30 times more rapidly during flight (Berger *et al*, 1971).

Assuming that a hummingbird spends only 25% of a 12 hour day flying (Hixon, 1982), and that EWL rate during flight were even 5 times the maximum resting rate measured by Powers (1992), then a 4.5 g hummingbird would lose 2.5 ml of water during

the 3 hour flying period each day. This is 125% of the amount estimated (Powers, 1992) for an entire day. Whatever the actual scenario, it is almost certain that field evaporative water losses are considerably higher than those measured in the lab by Laswieski (1964) and Powers (1992).

Conclusions

The findings from this study indicate that the hummingbird kidney does not eliminate the large volumes of water which have been suggested (Calder, 1979; Beuchat *et al*, 1991; Powers, 1992). Production of very large volumes of dilute urine would require an unusually large investment of energy in the kidney. The already large allocation of blood and energy to the kidney makes added investment unlikely, especially if extra-renal routes for water elimination can be used.

It is likely that the role of EWL in hummingbird water balance is even more important than suggested by Powers (1992). High EWL would not only serve as a route for water elimination, but also to enhance metabolic heat dissipation. Powers (1992) found that at best only 58.6% of metabolic heat was lost through evaporation. This lower than predicted (Calder and King, 1974) evaporative heat loss in hummingbirds "suggests that evaporative heat loss might not be as effective in hummingbirds as it is in other birds" (Powers, 1992). Alternatively, EWL rates in the field may be high enough that EWL is actually more effective for metabolic heat dissipation than predicted by laboratory measurements.

In addition, hydration state in hummingbirds is variable. This is clear from the rapid mass changes which occur immediately after dark and after the first morning feeding (Beuchat *et al*, 1979), as well as by the mass changes measured in the field (Powers and Nagy, 1988). Australian honeyeaters (Collins, 1981) also show this diurnal change in body mass. Although some of this change may be from fat metabolism and deposition, the rapid rates of change in the mornings and evenings suggest that much of it is due to change

in hydration state. The importance of this point is that hummingbirds do not have to maintain precise water balance from minute to minute, but from day to day. In fact, retention of some water during the day is probably important since the birds survive the night without feeding.

The hummingbird water load can be extremely variable. Metabolic requirements vary as a function of ambient temperature (Laswieski, 1963; Powers, 1992), which means that water loads will also vary. In addition, the magnitude of the water load is partially determined by variable nectar concentrations (Calder, 1979; Calder and Hiebert, 1983). Finally, the ability of the hummingbird to lose water evaporatively varies significantly with environmental temperatures and humidity (Powers, 1992). What is clear from this study is that water balance in hummingbirds is not a simple matter of matching renal excretion rate with water intake rate. Rather, water balance is almost certainly a dynamic process dependent on renal excretion, EWL, and temporary water retention.

Appendix

The following are the data plotted in Fig. 2. GFR values and masses from more than one study were averaged. Values listed in Roberts *et al* (1985) are here referenced under that citation. See Roberts *et al* (1985) for specific study citations. The values for the ostrich, *Struthio camelus*, are from individuals 4 - 6 weeks of age.

Acknowledgements — I would like to thank Kathryn Medler and the rest of my family for support and encouragement. I also thank those who discussed my methodology and those who critically read the manuscript. Thanks to Carol A. Beuchat for the opportunity to work on such an interesting project. This research was supported by a Sigma Xi research grant.

	Species	Mass (g)	GFR (ml/h)	Source
1.	*Calypte anna*	5.1	2.4	present study
2.	*Passer domesticus*	22.8	7.7	Goldstein and Braun (1988)
3.	*Melospiza melodia*	23.9[a]	12.6	Rothschild and Goldstein (1990)
4.	*Melopsittascus undulatus*	40.0	8.4	Roberts *et al* (1985)
5.	*Coturnix chinensis*	52.8	33.0	Roberts *et al* (1985)
6.	*Sternis vulgaris*	85.0	32.7	Roberts *et al* (1985)
7.	*Coturnix pectoralis*	103.4	40.8	Roberts *et al* (1985)
8.	*Zenaidura macroura*	110.0	16.2	Roberts *et al* (1985)
9.	*Coturnix coturnix*	125.0	71.1	Roberts *et al* (1985)
10.	*Callipepla gambelii*	156.5	14.7	Williams *et al* (1991)
11.	*Cacatua roseicapilla*	335.9	49.2	Roberts (1991)
12.	*Alectoris chuckar*	512.5	35.1	Goldstein (1990)
13.	*Larus glaucescens*	810.0	100.5	Roberts *et al* (1985)
14.	*Larus dominicanus*	905.0	185.0	Gray and Erasmus (1988)
15.	*Larus argentatus*	1000.0	276.0	Roberts *et al* (1985)
16.	*Anas platyrhynchos*	2162.0	352.0	Roberts *et al* (1985); Hughes *et al* (1989); Gerstberger *et al* (1985)
17.	*Gallus domesticus*	2180.0	296.4	Roberts *et al* (1985); Stallone and Braun (1985)
18.	*Branta canadensis*	3670.0	264.0	Roberts *et al* (1985)
19.	*Meleagris pavo*	7400.0	340.5	Roberts *et al* (1985)
20.	*Struthio camelus*	12200.0	683.0	Levy *et al* (1990)
21.	*Dromaius novaehollandiae*	34000.0	1109.3	Dawson *et al* (1985); Dawson *et al* (1991)

[a] Mass taken from Schluter and Smith (1986).

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Figure Legends

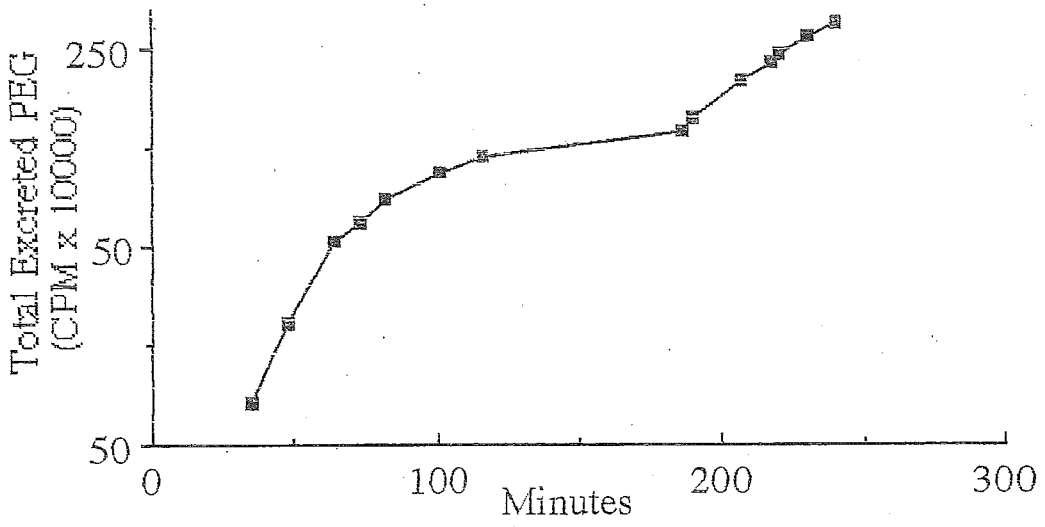
Fig. 1. ^{3}H-PEG excretion as a function of time from a clearance period not used for GFR determination. The rapid decline in excretion rate during the measurement is consistent with rapid GFR decreases. The hummingbird appeared to be going torpid during the period of apparent GFR shutdown.

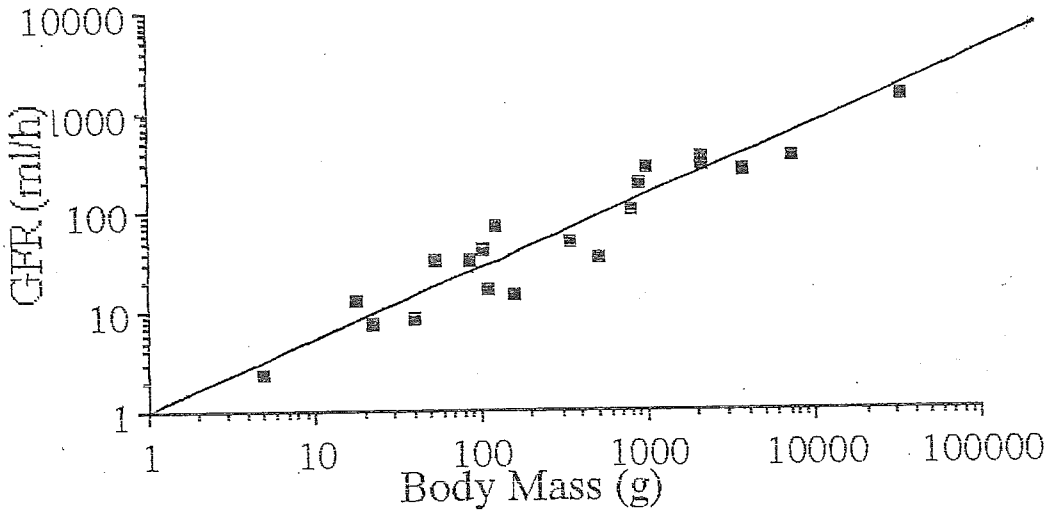
Fig. 2. GFR (ml/h) as a function of body mass (g) for 21 avian species, including the Anna's hummingbird. $GFR = 1.11\ M^{0.69}$; $R^2 = .91$. See appendix for sources.

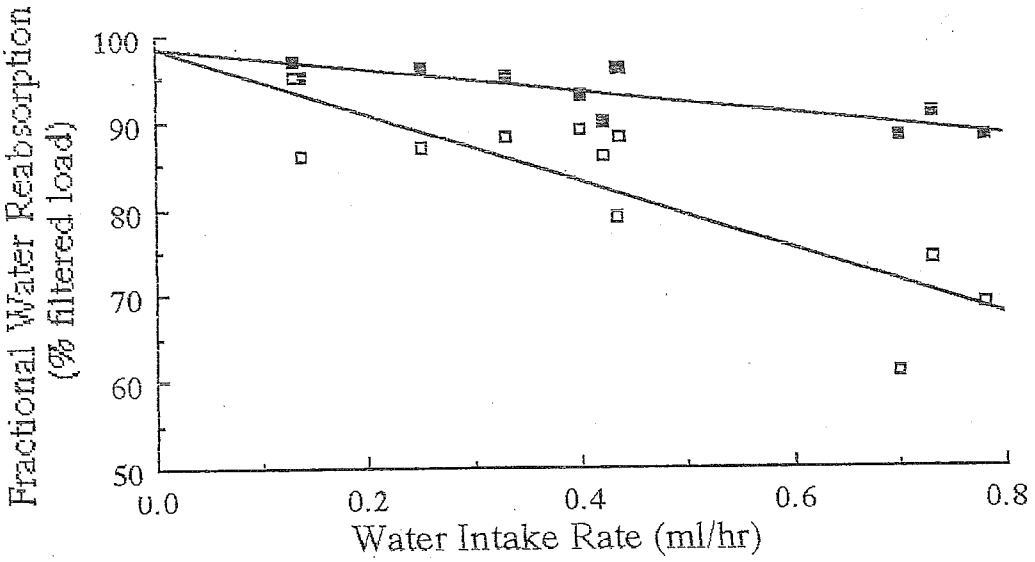
Fig. 3. Filled squares show measured FWR. FWR significantly decreased ($p < 0.005$) as water intake increased. The open squares show the fractional reabsorption needed in order to match renal water excretion rate with water intake rate.

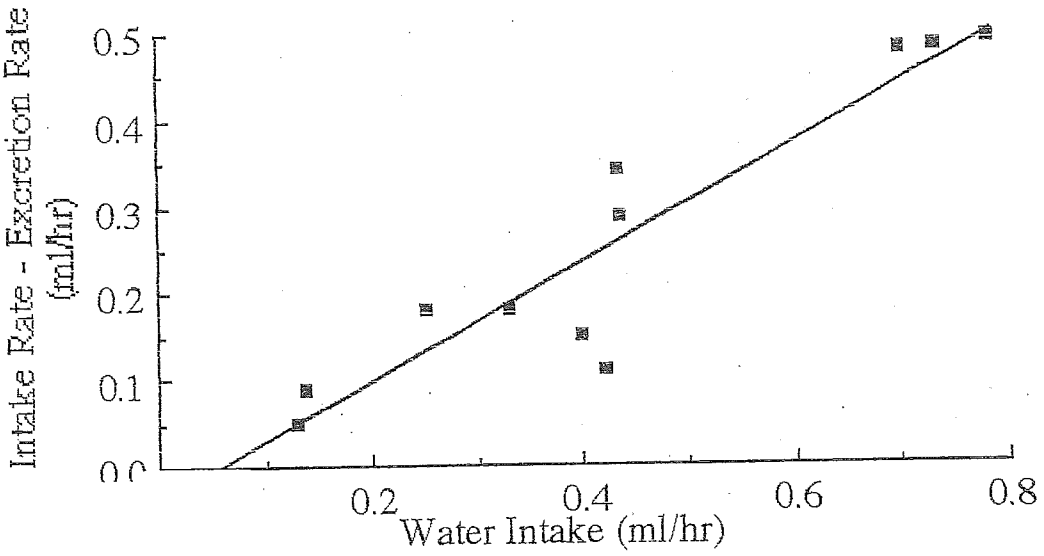
Fig. 4. The difference between water intake rate (ml/h) and water excretion rate (ml/h) significantly ($p < 0.0005$) increased as water intake increased. At a water intake rate of 7.2 ml/d, water intake rate is about .4 ml/h greater than excretion rate.

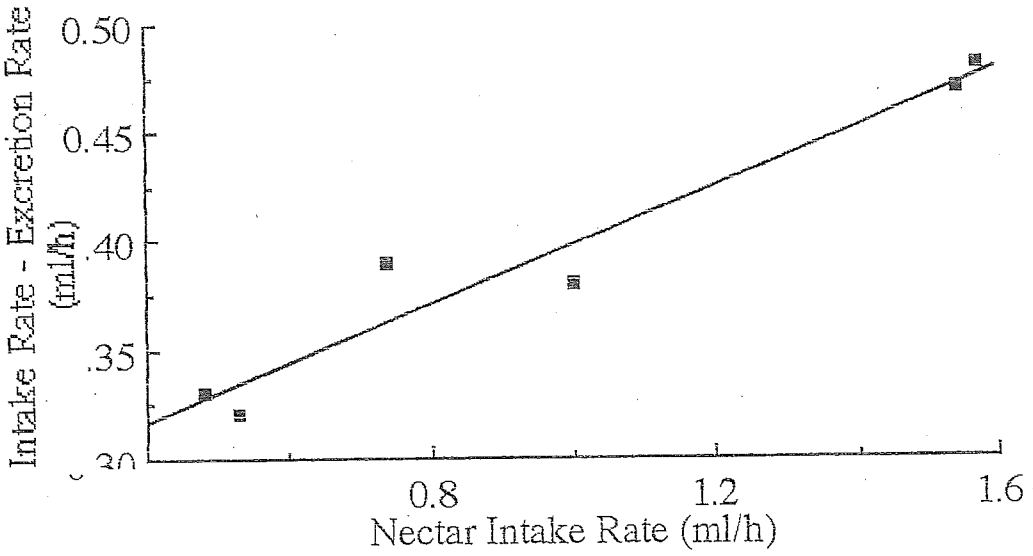
Fig. 5. The difference between nectar intake rate and water excretion rate in two Australian honeyeaters, *Lichmera indistincta* and *Acanthorhynchus superciliosis* (Collins 1981). As with the hummingbirds in this study, the difference significantly increased ($p < 0.005$) as nectar intake rate increased.


Total Excreted PEG
(CPM x 100000)
250
50
50
0
100
200
300
Minutes


10000
1000
100
10
1
GFR (ml/h)
1
10
100
1000
10000
100000
Body Mass (g)


Fractional Water Reabsorption
(% filtered load)
100
90
80
70
60
50
0.0
0.2
0.4
0.6
0.8
Water Intake Rate (ml/hr)


Intake Rate - Excretion Rate (ml/hr)
0.5
0.4
0.3
0.2
0.1
0.2
0.4
0.6
0.8
Water Intake (ml/hr)


Intake Rate - Excretion Rate (ml/h)
0.50
0.45
.40
.35
0.8
1.2
1.6
Nectar Intake Rate (ml/h)

J. theor. Biol. (1995) **176,** 59–65

Physiological Time and the Measurement of Glomerular Filtration Rate

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(*Received on* 30 *August* 1994, *Accepted in revised form on* 25 *April* 1995)

Diverse physiological timescales in different animals can have an important influence on the measurement of biological variables. In vertebrates, the absolute and relative rates of glomerular filtration span several orders of magnitude. These differences have important implications for the logistics of measurement protocols in different species. A simple model is used to examine the importance of physiological time to the measurement of glomerular filtration rate.

1. Introduction

The term physiological time describes the observation that different animals operate in an array of relative time scales (Calder, 1984). This relative time is manifested in virtually all aspects of an organism's existence, from expected lifespan to the time of a single cardiac cycle (Calder, 1984). Because these relative timescales so profoundly influence an animal's biology, the importance of physiological time to the application of measurement techniques also deserves consideration. Physiological measurements are performed on a variety of species that may differ greatly in body mass and phylogenic group. Two of the most important predictors of physiological time are body mass and phylogenic group (Calder, 1984). These differences should be recognized, and perhaps alternate measurement techniques should be applied in measuring physiological variables. The importance of physiological time should, therefore, be considered when initiating measurements on animals of different size or phylogeny. What follows is an examination of physiological timescale with respect to the measurement of glomerular filtration rate (GFR).

Glomerular filtration is a fundamental element in the processes that lead to osmotic homeostasis in vertebrates (Dantzler & Braun, 1980; Yokota *et al.*, 1985). The rate of filtration governs the amount and composition of fluid that will enter the nephron for processing. This central role of GFR in fluid homeostasis makes these measurements an important component in assessing and investigating renal function. In addition, GFR measurements may also be important to questions relating to other aspects of vertebrate biology such as ecology and energetics (see Beuchat *et al.*, 1991; Roberts, 1991).

The measurement of GFR in animals differing in phylogeny or body mass offers certain challenges that are not always obvious. Differences in physiological time mean that the percent of total plasma volume filtered per unit time differs drastically between different animals. This relationship arises because absolute filtration rates differ greatly between animals of different body size and phylogeny (Calder & Braun, 1983; Yokota *et al.*, 1985), while plasma volume tends to be a constant portion of body mass (Calder, 1984). GFR measurements on birds have been made on species spanning over three orders of magnitude in body mass. A 25 g bird has a GFR of about 0.20 ml min^{-1}, while a 1 kg bird has a GFR of about 2.35 ml min^{-1} (Williams *et al.*, 1991). However, the relative amount of plasma filtered is higher in the smaller animal since the total blood volume is equal to about 8% of body mass in mammals and birds (Calder, 1984). Assuming a hematocrit of around 40%, plasma volume is roughly 5% of an animal's total body mass. Thus, the 25 g bird will filter its entire plasma volume in just over 6 min. The larger 1 kg bird will require 21 min to filter the same proportion of its plasma. There are also large differences between phylogenic

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TABLE 1
GFR (ml hr^{-1}) as a function of body mass (g) in different vertebrate classes

Class	GFR (ml hr^{-1})	GFR (ml hr^{-1}) of 100 g animal	Number of species	Source
Mammals	1.24 M$^{0.765}$	42	41	Yokota *et al*., 1985
Birds	1.29 M$^{0.68}$	30	15	Williams *et al*., 1991
Reptiles	0.0058 M$^{0.999}$	0.58	22	Yokota *et al*., 1985
Amphibians	0.049 M$^{0.894}$	2.8	13	Yokota *et al*., 1985
Teleosts	0.010 M$^{0.788}$	0.38	15	Yokota *et al*., 1985

groups. Take for example, the differences between a 100 g mammal, bird, and reptile all having about a 5 ml plasma volume. The mammal will have a GFR of 0.70 ml min^{-1} (Yokota *et al*., 1985); the bird will have a GFR of 0.49 ml min^{-1} (Williams *et al*., 1991); and the reptile will filter 0.01 ml min^{-1} (Yokota *et al*., 1985). The time required to filter the total plasma volume will be 7 min for the mammal, 10 min for the bird, and 8.6 h for the reptile. These differences will create disparities in the dynamics of the variables involved in the measurement of GFR.

2. Relative Filtration Rate

The percent of total plasma volume filtered every minute will be referred to as the relative filtration rate (RFR). RFR can be measured directly or derived using estimates of GFR and estimates of total plasma volume. GFR can be estimated by using equations which describe absolute GFR as a function of body mass.

The relationships between GFR and body mass have been derived from selected species within the vertebrate classes (Table 1). GFR, as many functions, correlates with body mass in a logarithmic fashion:

$$GFR = aM^b. \quad (1)$$

Assuming that total plasma is 5% of body mass in all animals, and that the plasma has approximately the same density as water (1 g ml^{-1}), then RFR (% min^{-1}) can be related to body mass as:

$$RFR\ (\%/\text{min}^{-1}) = [GFR\ (\text{ml min}^{-1})/0.05\ \text{M (ml)}]\cdot 100\%. \quad (2)$$

By substituting the appropriate equations from Table 1, general relationships between RFR and body mass within a group of animals can be calculated (Table 2).

Note the differences in RFR which exist between animals of different body mass and phylogenic group. In all groups except for the reptiles, the exponential term is considerably less than zero (Table 2). A 1 kg chicken is predicted to have an RFR of 4.7% min^{-1}, while a 5 g hummingbird should have an RFR of 25.7% min^{-1}. Ectothermic vertebrates can be expected to have an RFR < 1, while most all mammals and birds will have an RFR > 1. The physiological significance of the differences in GFR between vertebrate groups is discussed elsewhere (Calder & Braun, 1983; Yokota *et al*., 1985). The importance for measurement purposes is that when RFR differs significantly between animals, the technical variables needed for GFR measurement must be adjusted accordingly.

3. Measurement of GFR

The determination of GFR involves the measurement of clearance of a filtration marker from the plasma. Clearance of a filtration marker is the volume of plasma completely cleared of the material per unit time. Clearance is described by the equation:

$$\mathring{V}_c = (C_u \cdot \mathring{V}_u)/C_p. \quad (3)$$

In this equation, C_u and C_p are the urinary and plasma concentrations of the fluid marker, respectively, and $\mathring{V}_u$ is the urinary flow rate (Brenner *et al*., 1986). If the marker is uncharged biologically inert, freely filtered at the glomerulus, and if the substance is neither secreted nor reabsorbed by the nephron, then the clearance of that marker is equal to the GFR (Brenner *et al*., 1986). While inulin is considered to be one useful filtration marker, there are a variety of other available compounds (Brenner *et al*., 1986).

The product of urinary marker concentration (C_u) and urinary flow rate ($\mathring{V}_u$), is equal to the marker

TABLE 2
RFR (% min^{-1}) as a function of body mass (g) in different vertebrate classes

Class	RFR (% filtered min^{-1})	RFR of 100 g animal
Mammals	41 M$^{-0.235}$	13.9
Birds	43 M$^{-0.32}$	9.9
Reptiles	0.19 M$^{-0.001}$	0.2
Amphibians	1.63 M$^{-0.106}$	1.0
Teleosts	0.33 M$^{-0.211}$	0.1

excretion rate ($\mathring{V}_{ex}$). This means that GFR is equal to $\mathring{V}_{ex}/C_p$. The dynamics of these two variables during a clearance period are largely determined by RFR. For example, the length of time from introduction of a plasma marker to the time of plasma marker stability, as well as the maintenance of such stability, are intimately related to RFR.

A simple mathematical model can be consulted to provide quantitative guidelines for measurements. The model also provides a conceptual framework for the measurement of GFR in animals of different body mass and phylogeny.

4. Mathematical Model

As a filtration marker is introduced into the circulation, the concentration in the plasma will begin to increase. At the same time, the substance will begin to be filtered at the glomerulus. The plasma concentration will increase until the rate of introduction and rate of elimination are equal. Once introduction rate and excretion rate are equal, the system will be in equilibrium until at least one of these rates changes. If either the introduction or excretion rate does change, then the system will reestablish a new equilibrium after a period of time. The length of that period is directly related to the RFR. If the RFR is high, then equilibrium will be established more quickly than if RFR is low.

Each minute the amount of the marker in the plasma, and therefore the plasma marker concentration, will be determined by four variables. These variables are the amount of marker introduced into the plasma during that minute, the amount of marker excreted during that minute, the amount of marker left in the circulation from the previous minute, and the total plasma volume (P_v). In turn, excretion rate will be the product of the total amount of marker in the plasma and the RFR. While changes in these variables actually occur continuously, it is convenient to break these processes into discrete events for modelling purposes. This means that logarithmic processes will be broken into a series of minute-by-minute linear steps. An example may best illustrate the basic nature of these relationships.

Assume an introduction rate of 1000 units of marker/min^{-1}, a plasma volume of 1 ml, and an RFR of 10% min^{-1}. During the first minute, 1000 units are introduced into the system. Before any marker is filtered, the plasma concentration is 1000 units ml^{-1}. After 10% of the marker is filtered, the total marker in the system is 900 units and the concentration is 900 units ml^{-1}. During the second minute, another 1000 units are introduced into the system and the total marker in the system is now 900 + 1000 or 1900 units. After filtration of 10% of the marker, 1710 units are left in the system. The amount of marker in circulation will increase each minute, until the excretion rate equals the introduction rate ($\mathring{V}_i$). The total amount of marker in the plasma (R_{total}) is defined by:

$$R_{total}(\text{units}) = \mathring{V}_{ex}(\text{units min}^{-1})/RFR(\%\ \text{min}^{-1}). \quad (4)$$

So once equilibrium is reached, the excretion rate will be 1000 units min^{-1}, and the amount of marker in the plasma will be 1000 (units min^{-1})/10% (min^{-1}), or 10 000 units.

The general format of this model system was written as a simple algorithm to increase the efficiency of the calculations. In this algorithm, the amount of marker in the circulation is defined in terms of three variables: the amount of marker in circulation at minute $x(R_x)$ can be defined in terms of the amount of marker in circulation at minute $x-1(R_{x-1})$; the amount of marker introduced into the system during minute $x(I_x)$; and the amount of marker excreted in minute x (E_x):

$$R_x(\text{units}) = R_{x-1}(\text{units}) + I_x(\text{units}) - E_x(\text{units}). \quad (5)$$

The amount of marker excreted during minute x is defined by:

$$R_x(\text{units}) \cdot RFR\ (\%\ \text{min}^{-1}). \quad (6)$$

During the first minute of our example:

$$R_1(\text{units}) = R_0(\text{units}) + I_1(\text{units}) - E_1(\text{units})$$

or

$$900(\text{units}) = 0(\text{units}) + 1000(\text{units}) - 100(\text{units}).$$

During the second minute:

$$R_2(\text{units}) = R_1(\text{units}) + I_2(\text{units}) - E_2(\text{units})$$

or

$$1710(\text{units}) = 900(\text{units}) + 1000(\text{units}) - 190(\text{units}).$$

A program written in BASIC implements this algorithm on a minute-by-minute basis. The figures and equations in the following discussion were generated using this fundamental program. The only assumptions in this model are that the plasma marker is introduced into the circulation and that a percentage of the total plasma volume is filtered per unit time. The model was used to examine issues related to plasma marker introduction and to departures from equilibrium during a measurement period.

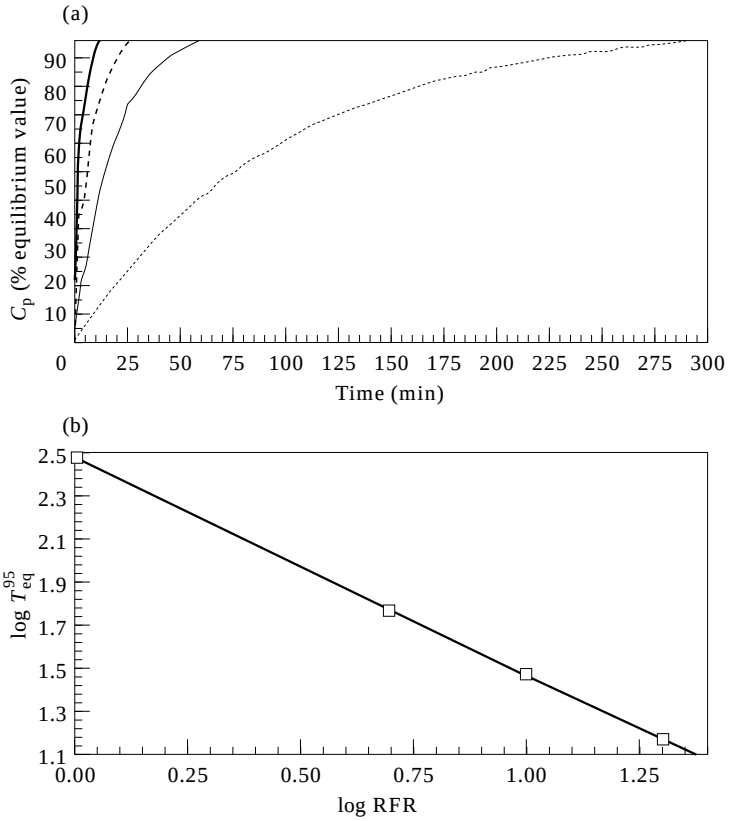

FIG. 1. (a) Plasma marker concentration (% equilibrium value) is plotted as a function of time (min). C_p approaches 95% of the equilibrium value at different rates, depending on RFR. The shape of the curves is the same; the effect of RFR is to either stretch or compress the curve on an absolute time scale. (b) Plotting log T_{eq}^{95} as a function of log RFR yields a straight line described by the general relationship: log $T_{eq}^{95} = 2.47 - (\log \text{RFR})$, or $T_{eq}^{95} = 295/\text{RFR}$. This relationship may be important during experimental design. RFR (%min^{-1}): (– – –) 1%, (——) 5%, (----) 10%, (——) 20%.

5. Introduction of a Filtration Marker

There are two important questions related to the introduction of the filtration marker. First is the length of time required after the initiation of marker introduction before the plasma concentration will begin to reach stability. Is there a relationship between the marker introduction rate and the time to reach a stable plasma concentration? Second is the amount of filtration marker that must be introduced in order to obtain a measurable plasma marker concentration. Enough marker must be introduced so that a measurable plasma concentration is obtained. However, using more marker than the amount required is not only wasteful, but could be harmful to the experimental animal.

The time needed for filtration marker concentration to reach stability in the plasma is related to the amount of time needed to reach equilibrium (T_{eq}) in the system. That is, marker concentration will become more stable as $\mathring{V}_{ex}$ approaches $\mathring{V}_i$. The time required for the system to reach equilibrium is inversely related to the RFR. Figure 1(a) shows the relationship between the time needed to reach equilibrium and RFR. The general shape of the plasma marker concentration curve through time is the same at any RFR. The initial concentration increases quickly as compared with the asymptotic approach to the equilibrium concentration. The effect of RFR on the concentration curve is to either stretch or compress the curve on an absolute time scale.

The relationship between T_{eq} and RFR can be described mathematically after plotting T_{eq} as a function of RFR. Since the plasma marker concentration curve approaches equilibrium asymptotically, T_{eq}^{95} may be defined as the plasma marker concentration at which 95% of the expected equilibrium is reached. The relationship between T_{eq}^{95} and RFR is shown in Fig. 1(b). The equation describing this relationship is:

$$T_{eq}^{95}\,(\text{min}) = 295/RFR(\%\ \text{min}^{-1}) \qquad (7)$$

Note that neither introduction rate nor the absolute amount of marker added into the circulation influence the T_{eq}^{95}. Instead, RFR is the fundamental determinant

of this parameter. T_{eq}^{95} is determined by the experimental animal and cannot be altered by the measurement technique employed. The relationship between RFR and T_{eq}^{95} is an inverse one. Therefore, large animals will require a longer period of time to reach equilibrium than small animals and ectothermic animals will require a much longer time period to reach equilibrium than endothermic animals.

The marker introduction rate needed for a measurable plasma concentration is influenced by three parameters. These are the RFR, P_v, and the total amount of plasma available for sampling. The plasma marker concentration can be expressed as R_{total} divided by P_v:

$$C_p(\text{units ml}^{-1}) = R_{total}(\text{units})/P_v(\text{ml}) \quad (8)$$

At equilibrium, the marker excretion rate and introduction rate are equal, so eqn (4) becomes:

$$R_{total}(\text{units}) = \dot{V}_i(\text{units min}^{-1})/RFR(\%\ \text{min}^{-1}) \quad (9)$$

Substituting this expression for R_{total} in eqn (8) shows that the plasma marker concentration at equilibrium is equal to:

$$C_p(\text{units ml}^{-1}) = [\dot{V}_i(\text{units min}^{-1})/ RFR(\%\ \text{min}^{-1})]/P_v(\text{ml}). \quad (10)$$

This equation reveals that the C_p is a direct function of the introduction rate. While RFR and plasma volume also determine plasma concentration, only introduction rate is under the control of the investigator.

As a practical example, osmotic minipumps have been successfully used as marker delivery devices in avian GFR measurements (Goldstein & Braun, 1988; Roberts & Dantzler, 1989; Rothschild & Goldstein, 1990; Williams *et al*., 1991; Goldstein &Rothschild, 1993). These studies generally cite the volume capacity and pumping rate, the amount or concentration of marker loaded in the pumps, and the time period between minipump implantation and GFR measurements. These papers report GFR measurements made on birds ranging from about 18 to 158 g in body mass. Suppose a comparative physiologist wanted to follow these protocols for GFR measurements in a comparably sized lizard (100 g). How should the experimental specifications be adjusted for this animal? The RFR of birds in this size range is expected to be from about 8.5 to 17% min^{-1}. The lizard is expected to have an RFR of about 0.19% min^{-1}. From eqn (10), the lizard will have a C_p that is about 45 to 90 times higher than the birds assuming everything else is equal. Thus, the $\dot{V}_i$ could be greatly reduced by lowering the concentration of loaded marker, resulting in the savings of an expensive marker. From eqn (7), the T_{eq}^{95} in the lizard will also be 45 to 90 times longer than in the comparable birds. There may be need to wait longer before measurements are taken.

6. Departures from Equilibrium

Any change in GFR, marker introduction rate, or plasma volume will create a departure from stability in excretion rate and plasma marker concentration. Modulation of GFR is an important point of osmoregulation in non-mammalian vertebrates (Braun & Dantzler, 1972; Calder & Braun, 1983; Yokota *et al*., 1985; Dantzler, 1988; Goldstein & Rothschild, 1993); taking plasma samples for marker concentration will change the plasma volume; and marker introduction rate may not be perfectly constant.

The rate of change that occurs in the variables used to estimate GFR during a non-equilibrium state is inversely proportional to RFR. This relationship means that there are practical limitations to the measurement of plasma marker concentration and marker excretion rate which are related to RFR; and that different animals have different inherent variability in marker concentration and excretion rate depending on their RFR. Two examples illustrate these trends. The first of these uses the single injection method for measuring GFR and the second uses a hypothetical GFR change.

The single injection method does not use a continuous introduction of filtration marker as in standard clearance techniques. Instead, a single bolus of marker is introduced directly into the circulation. Because the filtration marker introduction rate is zero, the marker will immediately begin to disappear from the plasma. The higher the RFR, the more quickly the rate of marker disappearance. The ratio between the amount of marker introduced and the area under the plasma marker disappearance curve is used to estimate GFR (Mitch & Walser, 1986).

Figure 2 shows the relationship between RFR and plasma marker disappearance curves. Plotting the time needed to reach 5% of the initial plasma marker concentration shows that this time is the same needed to reach 95% T_{eq}. That is, the time needed to reach the 5% level ($T_{5\%}$) is described by the same relationship as shown in eqn (7):

$$T_{5\%}(\text{min}) = 295/RFR(\%\ \text{min}^{-1}). \quad (11)$$

An animal with an RFR of 1% will reduce plasma marker concentration to 5% of the original concentration in 300 min. In contrast, an animal with

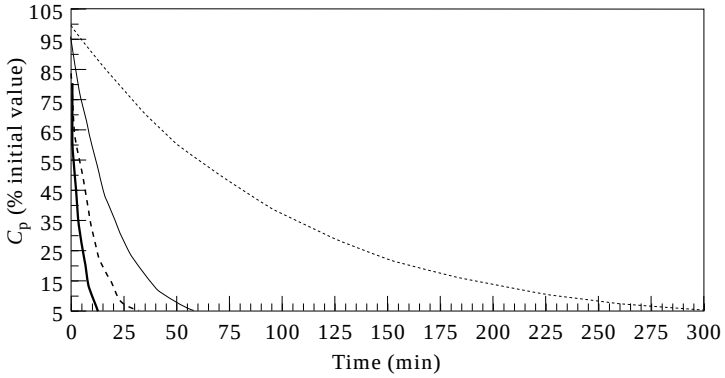

FIG. 2. Plasma marker concentration (% initial value) is plotted as a function of time (min) as in a single injection measurement of GFR. Since marker introduction rate is zero, the marker begins to leave the plasma at the start of the experiment. The rate at which the marker concentration approaches 5% of the initial value is dependent on RFR: the higher the RFR, the higher the rate of marker disappearance. RFR (%min^{-1}): (– – –) 1%, (——) 5%, (----) 10%, (——) 20%.

an RFR of 20% will reach the same level in less than 15 min.

As in single injection measurements, any changes in filtration marker concentration will occur at a rate inversely proportional to RFR. Figure 3(a) shows a hypothetical 18% reduction in equilibrium GFR and back again over a 40 min time period. Figure 3(b) shows how plasma levels (% of equilibrium value) change in response to this change in GFR. An animal with a higher RFR will have a marker concentration that is inherently more labile on an absolute time-scale than an animal with a lower RFR. Transient

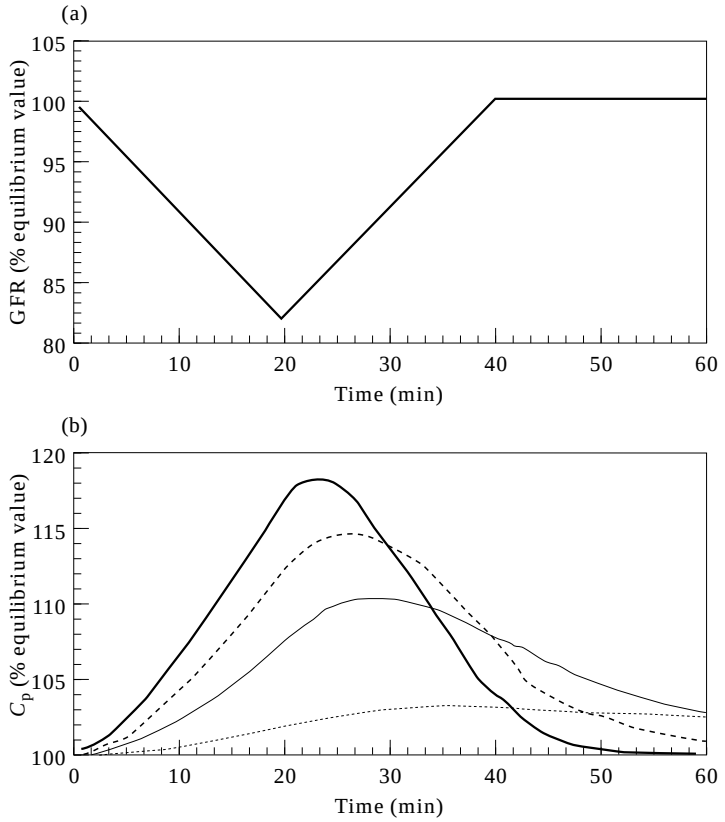

FIG. 3. (a) GFR (% equilibrium value) is plotted as a function of time (min). This hypothetical example shows a reduction to 18% of equilibrium GFR and back again in a 40 minute period. (b) Plasma marker concentration (% equilibrium value) plotted as a function of time (min) changes as a result of the GFR change in (a). The same relative change in GFR leads to disproportionate relative changes in plasma marker concentration. Notice that the changes in plasma marker concentration more closely follow the GFR change when RFR is high. RFR (%min^{-1}): (– – –) 1%, (——) 5%, (----) 10%, (——) 20%.

increases or decreases in GFR may be especially problematic. Such temporary changes in GFR can cause dramatic changes in plasma marker concentration which quickly return to their previous levels. Concomitant changes in marker excretion rate may be missed, since excretion rate is measured over a time continuum.

The avian GFR protocols using minipumps (Goldstein & Braun, 1988; Roberts & Dantzler, 1989; Rothschild & Goldstein, 1990; Williams *et al*., 1991; Goldstein & Rothschild, 1993) can exemplify the potential for measurement error. In the laboratory, one method for estimating excretion rate is by collecting total excreted marker over 3 or more hours (Goldstein & Braun, 1988; Roberts & Dantzler, 1989; Williams *et al*., 1991; Goldstein & Rothschild, 1993). In field studies (Rothschild & Goldstein, 1990; Goldstein & Rothschild, 1993) excretion rate is assumed to be equal to manufacturer's specified pump rate, given that equilibrium has been reached. In all cases, GFR measurements are based on either a mean excretion rate or an estimated excretion rate. In contrast, blood samples are taken at a single point in time at the end of the experimental period. Since modulation of GFR is an important point of regulation in some animals, it is likely that the stress of blood sampling affects GFR. When this occurs, the calculated GFR does not represent the experimental GFR, but largely reflects the effects of handling. Again notice that the departures from equilibrium are strongly influenced by an animal's RFR (Fig. 3). At least one study (Rothschild & Goldstein, 1990) dealt with this problem by taking blood samples within two minutes of recapture.

7. Conclusion

Differences in physiological time between animals of distinct phylogeny and body mass are intimately related to the practical measurement of GFR. This paper has been an attempt to discuss some of the most basic elements of any GFR measurement. Even at this most basic level, some trends may seem unexpected or even counter-intuitive, such as the fact that T_{eq} is determined by RFR and not by the rate of marker introduction.

It is important to tailor the appropriate measurement protocol to the experimental animal. The protocol used to measure GFR in a reptile would be of little use to an investigator attempting to measure GFR in a small bird or mammal. The examination of quantitative relationships can serve as a conceptual framework for planning purposes. While examination of these relationships does not replace experimentation, examination of general relationships can complement experimentation. What is presented here is but an example of the need to consider physiological timescale when planning physiological measurements. There are almost certainly a number of other experimental procedures greatly influenced by differences in physiological time and this influence should be carefully considered when initiating measurements.

I am very grateful to all of those who took time to review this paper and make critical suggestions.

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