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Fortification of an Aquafeed with Potassium Chloride Does Not Improve Survival of Juvenile Australian Snapper *Pagrus auratus* Reared in Potassium Deficient Saline Groundwater

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Abstract: This study was done to determine if fortification of a commercial aquafeed with KCl could improve the survival of juvenile Australian snapper *Pagrus auratus* reared in K^+ deficient saline groundwater (KDSGW; <5 mg K^+ L^{-1}). Experiment 1 (Exp. 1) tested whether feeding an aquafeed fortified with zero, 25, or 50 g KCl kg^{-1} for 6 days affected feed intake and survival of fish transferred immediately from estuarine water to KDSGW of the equivalent salinity (20 g·L^{-1}). Experiment 2 (Exp. 2) investigated whether an aquafeed fortified with zero, 10, or 25 g KCl kg^{-1} affected survival, feed intake, and growth rate (SGR) of snapper reared in KDSGW fortified to have 40% or 100% the [K^+] of equivalent salinity estuarine water (20 g·L^{-1}). The results of Exp. 1 demonstrated there was no benefit of fortifying aquafeed with KCl; fish transferred into KDSGW stopped feeding and developed symptoms akin to tetany. Some individuals also died and others became moribund. Exp. 1 was terminated according to animal care and ethics guidelines. The results of Exp. 2 indicated the amount of KCl added to the aquafeed did not affect survival, feed intake, or food conversion ratio (FCR) of snapper, irrespective of water treatment. However, SGR and FCR was better when fish were reared in normal estuarine water and KDSGW fortified to have 100% the [K^+] of equivalent salinity estuarine water. Our results demonstrated that juvenile snapper were unable to utilize the KCl added to the aquafeed and were probably reliant on sequestering K^+ ions from the water column in order to maintain functions involving hydromineral homeostasis. Fortification of aquafeeds with KCl does not ameliorate the negative effects of KDSWG on the survival of juvenile snapper.

Keywords: saline groundwater; mineral deficiency; red sea bream; hydromineral balance; KCl

1. Introduction

In Australia, rising water tables have carried large amounts of salt to the surface, damaging infrastructure and reducing the fertility and productivity of agricultural land [1–3]. In some areas such as the Murray-Darling Basin (MDB), this problem is ameliorated by large interception schemes which pump saline groundwater from subterranean aquifers into evaporation basins [2]. The potential use of interception schemes for mariculture is considerable [1,4–8], but in many cases this use is limited by sub-optimal ionic composition [2,9]. The ionic composition of groundwater following evaporation can also vary considerably depending on local geology, climate, and surface management [10]. Irrespective of these factors, the majority of saline groundwater sources in Australia are deficient in K^+, compared with seawater [1,2,5].

Under normal circumstances, marine and euryhaline fish are capable of meeting their ionic and osmotic requirements for most minerals from the water in which they live [11–13]. Such species have evolved sophisticated mechanisms of hydromineral balance that regulate the major ions in seawater [14] and this regulation is mainly achieved by the cooperative function of the gills, intestine, and kidney [13,15–17]. The gastrointestinal tract (GI tract) of marine fish plays a critical role in osmoregulation via the process of NaCl coupled fluid absorption [18–21]. However, the dual role of the intestine, serving hydromineral functions as well as the digestion and absorption of nutrients, is often in conflict and many of the interactions are not well understood [17–19,22–25]. It is known that consumption of large meals of dry aquafeed immediately increase the load of minerals in the GI tract, drastically changing the ionic balance of the intestine [21,24].

Numerous studies have indicated that extreme imbalances in the ionic composition of water effect hydromineral balance in fish and shrimp resulting in low survival and poor health [3,26–30]. When a K^+ deficiency occurs, fish display symptoms such as poor growth, elevated food conversion ratio (FCR), anorexia, convulsions, tetany, and weakness or failure of skeletal, cardiac, and respiratory muscles [11,31]. These responses indicate a clear dependency on K^+ for regulating body fluid homeostasis and salt secretion, see [13,16,32–35]. Fielder [5], Partridge and Creeper [28], and Doroudi et al. [3] have already demonstrated that morbidity and mortality of euryhaline/marine species such as snapper, Barramundi *Lates calcarifer*, and mulloway *Argyrosomus japonicus* reared in KDSGW can be overcome by the direct addition of KCl to the water, achieving near optimal growth when the $[K^+]$ of water was elevated above 60% the concentration of equivalent salinity seawater.

The mariculture potential of interception schemes associated with the MDB is tempered by the high cost of ameliorating these water bodies with potassium salts [9]. For this reason, there has been much interest in the potential of including additional K^+ in aquafeeds. Nonetheless, studies considering the impact of feeding on hydromineral balance in fish are rare. Even rarer are studies considering the impacts of feeding when there is an ionic deficiency in the rearing medium. Fish reared in freshwater where $[K^+]$ was low were shown to have some requirement for dietary K^+ [31,36–38]. However, evidence that marine fish respond positively to dietary salts let alone dietary K^+ remains a point of conjecture, mostly because studies have been conducted in water bodies where $[K^+]$ and the concentration of other ions was probably sufficient to meet their needs, e.g., [39]. Studies with Barramundi showed that the addition of spring salt to their feed actually reduced growth and increased FCR when they were reared in low-salinity brackish water [40]. In contrast, gilthead seabream *Sparus aurata* reared in low-salinity brackish waters responded positively to high additions of NaCl as evidenced by better survival and growth rate [41]. Cobia *Rachycentron canadum* reared at low salinity recorded an increase in feed intake and worsening FCR in response to feeds supplemented with increasing levels of NaCl [42]. The effects on cobia were attributed to lower energetic cost of osmoregulation at intermediate levels of NaCl supplementation due to increased proliferation of gill chloride cells and reduction in gill Na^+-K^+ ATPase activity [42]. Dietary supplementation of NaCl was effective in improving FCR and weight gain of the euryhaline red drum *Sciaenops ocellatus* when the fish were reared in fresh or brackish-water, but not when the fish were reared in seawater [43].

Potassium deficient groundwater is suitable for culture of penaeids if the water or the feed is fortified with K^+ [26,44–49]. However, the benefit of feeding K^+ to shrimp also appears equivocal as some authors have shown that shrimp *Litopenaeus vannamei* reared in artificial seawater, where the $[K^+]$ of water and feeds were varied, did not gain any substantial benefit from K^+ fortified feed [8]. Additionally, the supplementation of K^+ and Mg^{2+} in feeds at levels above normal dietary requirements did not appear to benefit *L. vannamei* reared in low salinity waters where the ionic composition was considered stressful [50]. Several studies have indicated that addition of K^+ or chelated K^+ to feeds results in a minor improvement in the weight gain of shrimp in the laboratory [8,48], but these results have not been reproduced at the farm level. Much of the research on shrimp implicates the specific ratio of Na:K in the rearing medium as being one of the most important factors affecting survival

and growth, apparently due to the profound effect that the Na:K ratio as well as the osmolality of the culture medium has on the activity of Na^+-K^+-ATPase, see [7].

The aim of this study was to determine if the addition of K^+ as KCl to a commercial aquafeed could improve the short term survival, feed intake, and growth rate of juvenile snapper reared in KDSGW obtained from the MDB interception scheme.

2. Results

2.1. Addition of KCl to the Commercial Aquafeed

Addition of KCl to the commercial aquafeed resulted in minor changes to some of the proximate components (Table 1). The highest level of KCl supplementation resulted in five- and three-fold increases in K^+ and Cl^-, respectively, compared with the unfortified aquafeed. There were minor reductions in the P and Fe content of the aquafeed as the level of KCl increased (Table 1).

Table 1. Analysed composition of unfortified aquafeed and the same aquafeed fortified with 10, 25 or 50 g KCl kg^{-1} (as fed basis).

Parameter		Aquafeed (No KCl Added)	Aquafeed +10 g KCl kg^{-1}	Aquafeed +25 g KCl kg^{-1}	Aquafeed +50 g KCl kg^{-1}
Moisture	%	66.6	53.4	36.4	56.2
Crude protein	%	517.0	520.0	527.0	502.0
Ash	%	96.3	103.0	120.0	137.0
Fat	%	131.0	136.0	159.0	103.0
Energy ¥	($MJ \cdot kg^{-1}$)	20.6	20.9	21.4	19.4
Cl^-	($g \cdot kg^{-1}$)	6.4	10.7	17.2	20.8
Ca^{2+}	($g \cdot kg^{-1}$)	23.9	26.5	26.5	24.9
Mg^{2+}	($g \cdot kg^{-1}$)	1.5	1.5	1.5	1.4
Na^+	($g \cdot kg^{-1}$)	4.1	4.1	4.2	4.0
K^+	($g \cdot kg^{-1}$)	4.9	10.2	16.3	27.5
P	($g \cdot kg^{-1}$)	31.9	16.0	16.0	15.1
Fe	($mg \cdot kg^{-1}$)	537.0	467.0	473.0	437.0
Cu	($mg \cdot kg^{-1}$)	11.0	10.5	10.5	11.9
Zn	($mg \cdot kg^{-1}$)	169.0	171.0	173.0	169.0
I	($mg \cdot kg^{-1}$)	3.3	7.5	7.5	7.5
Na:Cl		0.6	0.4	0.2	0.2
Na:K		0.8	0.4	0.3	0.2
Ca:Mg		15.9	17.7	17.7	17.8
Ca:P		0.7	1.7	1.7	1.7

¥ Gross energy of diets estimated from energy equivalents cited for protein, fat, ash and nitrogenous free extractives [51].

2.2. Manipulation of K^+ Deficient Saline Groundwater (KDSGW) and Estuarine Water Treatments

The $[K^+]$ of full strength KDSGW from Wakool was approximately 22 $mg \cdot L^{-1}$. After dilution with rainwater to a nominal salinity of 20 $g \cdot kg^{-1}$, the $[K^+]$ decreased to approximately 5.0 $mg \cdot L^{-1}$ (Table 2). Estuarine water adjusted to a salinity of 20 $g \cdot kg^{-1}$ had a $[K^+]$ of approximately 213 $mg \cdot L^{-1}$ which accurately reflected the $[K^+]$ of typical seawater diluted to the same salinity (e.g., $18.9/35 \times 399 = 215$ mg K^+ L^{-1}; Table 2). Measured $[K^+]$ of the fortified KDSGW treatments used in Exp. 2 indicated they were close to the targeted values of 40% and 100% the $[K^+]$ of equivalent salinity estuarine water (i.e., calculated as 41.4% and 96.6%), respectively. Differences in the average ionic composition of water treatments was exemplified by a 98%, 19%, and 16% decrease in the concentration of K^+, Na^+, and $SO_4{}^{2-}$, respectively, and an 18%, 32%, 153%, and 184% increase in concentration of Cl^-, Mg^{2+}, Ca^{2+}, and $HCO_3{}^-$, respectively, in dilute KDSGW compared to dilute estuarine water (i.e., at a salinity of 20 $mg \cdot L^{-1}$).

Table 2. Water chemistry of typical seawater and of estuarine water or K^+ Deficient Saline Groundwater (KDSGW) obtained from NSW DPI Inland Saline Aquaculture Research Institute at Wakool; as used in Exp. 1 and Exp. 2.

Parameter	Reference Waters		Raw Source	Experiment 1			Experiment 2			
	Seawater ¥	KDSGW ‡	KDSGW	Estuarine	Estuarine	KDSGW	Estuarine	Estuarine	KDSGW40 ¥¥	KDSGW100 ¥¥
pH	8.1	7.9	7.7 ± 0.2	8.4 ± 0.4	7.4 ± 0.1	8.0 ± 0.4	7.9 ± 0.2	7.7 ± 0.2	7.2 ± 0.1	7.1 ± 0.1
Salinity ($g \cdot L^{-1}$)	35	19.6	78.7 ± 0.4	23.5 ± 0.9	18.7 ± 0.7	19.7 ± 0.0	30.7 ± 1.4	19.1 ± 0.9	19.2 ± 0.1	18.2 ± 0.3
K^+ ($mg \cdot L^{-1}$)	399	9.2	21.9 ± 0.1	245.0+38.2	209.0+26.0	4.9 ± 0.3	349.0+15.6	217.5+24.7	90.0 ± 2.8	210.0 ± 1.0
Element or ion ($mg \cdot L^{-1}$)										
Cl^-	19,354	11,000	49,163	12,994	10,341	12,306	16,976	10,562	12,349	12,516
Na^+	10,770	4210	18,816	7231	5754	4710	9447	5877	4591	4351
$SO4^{2-}$	2712	1100	4916	1821	1449	1231	2379	1480	1199	1137
Mg^{2+}	1290	820	3664	866	689	917	1132	704	894	847
Ca^{2+}	412	504	2252	277	220	564	362	225	550	521
HCO^{3-}	142	195	872	96	76	218	125	78	213	202
Br^-	67	34	152	45	36	38	59	37	37	35
F^-	1.3	0.4	2.0	0.9	0.7	0.5	1.1	0.7	0.5	0.5
B	4.5	0.4	1.7	3.0	2.4	0.4	3.9	2.5	0.4	0.4
Sr^{2+}	7.9	9.1	40.7	5.3	4.2	10.2	6.9	4.3	9.9	9.4
Na:Cl	0.6	0.4	0.4	0.6	0.6	0.4	0.6	0.6	0.4	0.4
Na:K	27.0	457.6	859.2	29.5	27.5	961.2	27.0	27.0	51.0	20.7
Ca/Mg	0.3	0.6	0.6	0.3	0.3	0.6	0.3	0.3	0.6	0.6

¥ Composition of typical seawater [14]; ‡ Comparative data reproduced from [5]; ¥¥ Targeting 40% and 100% the [K^+] of equivalent salinity estuarine water.

The Na:Cl, Na:K, and Ca:Mg ratios of dilute estuarine water were approximately 0.56:1, 27:1, and 0.32:1, respectively, whereas the ratios of the same ions in unfortified KDSGW were 0.38:1, 961.23:1, and 0.61:1, respectively. Fortification of dilute KDSGW with the highest addition of KCl shifted the Na:K ratio of diluted KDSWG towards that of typical seawater or estuarine water, but the Na:K ratio of the 40% fortified KDSGW treatment was almost double that of similar salinity estuarine water (Table 2).

2.3. Exp. 1: Effect of KCL Fortified Aquafeed on Direct Transfer of Fish to K^+ Deficient Saline Groundwater KDSGW

No fish died and all fish ate vigorously during the first 6 days of Experiment 1. However, there was a significant decrease in the relative feed intake of fish offered the aquafeed fortified with KCl compared to fish offered the unfortified treatment during this period ($F_{2,15} = 16.6$, $p < 0.0002$; Table 3). Fish that were fed the 25 and 50 g KCl kg^{-1} treatments consumed 27% and 28% less feed, respectively, compared to the fish that were offered the unfortified aquafeed.

Table 3. Performance and survival of Australian snapper fed an aquafeed fortified with KCl prior to direct transfer to estuarine water or K^+ Deficient Saline Groundwater (KDSGW) of equivalent salinity (20 $g{\cdot}L^{-1}$; Exp. 1).

	Dietary KCl Concentration (g KCl kg^{-1})			
Performance Index	Residual	25	50	Pooled SEM
Acclimation feeding period (n = 6)				
Feed intake (% BW day^{-1})	2.34 [a]	1.71 [b]	1.67 [b]	0.09
Estuarine treatment only (n = 3)				
Survival (%)	100.00	100.00	100.00	na
Stock weight ($g{\cdot}fish^{-1}$)	22.49	22.77	22.16	0.51
Final weight ($g{\cdot}fish^{-1}$) [†]	24.81	24.09	22.79	0.67
SGR (% day^{-1})	1.22 [a]	0.70 [ab]	0.35 [b]	0.15
Feed intake (% BW day^{-1})	2.17 [a]	1.64 [b]	1.72 [b]	0.10
Feed conversion ratio [‡]	1.86	2.50	5.76	1.05
KDSGW treatment [¥] (n = 3)				
Survival (%)	70.8	79.17	83.33	12.50
Stock weight ($g{\cdot}fish^{-1}$)	22.26	22.66	22.74	0.26
Final weight ($g{\cdot}fish^{-1}$) [†]	23.05	21.68	22.15	0.41

[†] Final weight of surviving fish from each treatment. [‡] FCR data were log transformed prior to ANOVA to transform heterogeneous variances; however untransformed data is presented in the table. Different superscript letters within a row indicate a significant difference when $p < 0.05$. [¥] These data were not subjected to statistical analysis due to acute morbidity and mortality.

There were no obvious signs of distress in fish immediately following transfer to the estuarine or KDSGW treatments and most fish consumed some feed during the afternoon meal. Within 24 h of being transferred to KDSGW, 6 fish had died, however mortality was not related to any specific dietary treatment. The remaining fish in KDSGW appeared distressed and exhibited a fixed mouth gape (i.e., tetany; lock-jaw), and some were haemorrhaging around the mouth. At this stage, they were still responsive to external stimuli (e.g., tapping lightly on the tank wall) but they did not eat. As a consequence of increasing morbidity, all KDSGW units were immediately fortified with 20 g KCl to prevent further loss of fish and to adhere to animal care and ethics guidelines (i.e., ≈28 h after transfer). Prolonged exposure of the fish to the KDSGW would likely have resulted in 100% mortality. Recovery of the surviving fish was relatively rapid, signs of tetany declined, and the fish regained an interest in feeding (≈2–3 h post KCl fortification). Normal vigour and feeding behaviour was evident in all surviving fish the following morning. No mortality, loss of appetite, or abnormal behaviour was recorded in fish reared in the estuarine controls.

Due to the negative outcomes for fish transferred into KDSGW, these data were excluded from our a-priori statistical evaluation. Instead, a one-way ANOVA was used to assess the effect of KCl inclusion on SGR, relative feed intake, and FCR of fish held only in estuarine water (n = 3). These tests indicated there was a significant difference among the SGR ($F_{2,6}$ = 8.61, p = 0.017) and relative feed intake of fish fed different levels of KCl ($F_{2,6}$ = 8.20, p = 0.019), however there was no difference in FCR ($F_{2,6}$ = 3.92, p = 0.081) (Table 3). The SGR and relative feed intake were higher, respectively, in fish fed the unfortified diet than for fish fed the aquafeed containing 25 and 50 g KCl kg^{-1} (Table 3).

2.4. Exp. 2: Effect of KCl Fortified Aquafeed on Transfer of Fish to Fortified K^+ Deficient Saline Groundwater (KDSGW)

All fish survived stage 1 of Experiment 2. Two outliers, one each from the estuarine control and the 10 g KCl kg^{-1} treatments, were removed from statistical analysis of SGR prior to ANOVA as they were more than 3× the interquartile range. ANOVA indicated there were no differences among interim weight ($F_{2,24}$ = 0.41, p = 0.667), SGR ($F_{2,22}$ = 0.07, p = 0.933), relative feed intake ($F_{2,24}$ = 0.39, p = 0.684), or FCR ($F_{2,24}$ = 0.55, p = 0.584) of fish fed diets fortified with zero, 10, and 25 g KCl kg^{-1} (Table 4).

Table 4. Performance and survival of Australian snapper fed an aquafeed fortified with KCl and reared in undiluted estuarine water with a salinity of 31 $g{\cdot}L^{-1}$ for 13 days (Exp. 2; stage 1; n = 9).

		Dietary KCl Concentration (g KCL kg^{-1})		
Performance index	Residual	10	25	Pooled SEM
Survival (%)	100.00	100.00	100.00	na
Stock weight ($g{\cdot}fish^{-1}$)	37.33	37.46	37.83	0.21
Interim weight ($g{\cdot}fish^{-1}$)	45.64	44.46	45.10	0.51
SGR (% BW day^{-1}) [¥]	1.44	1.39	1.35	0.05
Feed intake (% BW day^{-1})	1.87	1.93	1.85	0.06
Feed conversion ratio	1.31	1.38	1.38	0.06

[¥] One outlier from the unfortified treatment and one from the 10 g KCl kg^{-1} treatment were excluded from ANOVA on SGR because they exceeded 3× the interquartile range; n = 8 for these treatments. There was no significant difference among treatments with respect to any performance index.

ANCOVA indicated that the covariate (i.e., interim weight; $F_{1,17}$ = 9.16, p = 0.008) and water treatment ($F_{2,17}$ = 12.17, p = 0.0004) significantly affected the harvest weight of fish at the end of stage 2. However, there was no effect of KCl level ($F_{2,17}$ = 0.57, p = 0.578) or the interaction of KCl level and water treatment ($F_{4,17}$ = 0.49, p = 0.741) on the harvest weight (Table 5). ANCOVA on SGR, relative feed intake, and FCR indicated the covariate was non-significant, so each model was reduced to a two-way ANOVA. The type of water treatment significantly affected the SGR of the fish ($F_{2,18}$ = 14.41, p = 0.0002), however, SGR was not affected by the level of KCl ($F_{2,18}$ = 0.61, p = 0.556) or the interaction between the level of KCl and the water treatment ($F_{4,18}$ = 0.63, p = 0.644). Relative feed intake was not significantly affected by the water treatment ($F_{2,18}$ = 1.56, p = 0.238), level of KCl ($F_{2,18}$ = 0.97, p = 0.396), or the interaction of these factors ($F_{4,18}$ = 0.84, p = 0.519). Feed conversion ratio (FCR) was significantly affected by water treatment ($F_{2,18}$ = 5.10, p = 0.017), but not by the level of KCl ($F_{2,18}$ = 0.72, p = 0.500) or the interaction of factors ($F_{4,18}$ = 0.75, p = 0.572) (Table 5). Five fish, each from different aquaria, died during the second stage of experiment 2. Mortality was not related to treatment effects.

Table 5. Performance of Australian snapper fed an aquafeed fortified with KCl and reared in estuarine water or KDSGW fortified to 40% and 100% [K^+] of equivalent salinity estuarine water (Exp. 2; 21 days; stage 2; n = 9).

	Harvest Weight ‡ (g·fish^{-1})	SGR (%BW day^{-1})	Feed Intake (%BW day^{-1})	FCR	Survival (%)
Water-type ¥					
Estuarine	55.93 ^{b}	1.06 ^{b}	1.74	1.65 ^{a}	98.15
40%	52.16 ^{a}	0.72 ^{a}	1.70	2.85 ^{b}	96.29
100%	57.97 ^{b}	1.23 ^{b}	1.84	1.52 ^{a}	96.29
Diet (g KCl kg^{-1})					
0	54.96	0.97	1.73	2.11	94.44
10	55.03	0.97	1.73	2.21	96.29
25	54.97	1.07	1.82	1.69	100.00
Pooled SEM	0.80	0.07	0.06	0.32	2.40

‡ Data on harvest weight are adjusted mean ± SEM according to the results of ANCOVA. ¥ All water sources were adjusted to a salinity of 20 g·L^{-1}. Significant differences ($p < 0.05$) between the level means of each factor are indicated by different superscript letters in each column.

3. Discussion

The results of Exp. 1 demonstrated that feeding juvenile snapper an aquafeed fortified with 25–50 mg KCl kg^{-1} did not ameliorate for the direct transfer of fish into KDSGW. Within 20 h of transfer, juvenile snapper exhibited symptoms typical of those reported for K^+ deficiency [11,29] and several fish died before the experiment was terminated. Our observations are reflective of reports on teleosts where K^+ had been accidently omitted from artificial seawater [29] and mirror those reported by Fielder et al. [5]. Our results are consistent with studies on other euryhaline species such as mulloway [3] and Barramundi reared in KDSGW [28]. In the case of Barramundi, transfer to KDSGW caused the degeneration and necrosis of skeletal muscle, hyperplasia of mitochondria rich cells (MRC or ionocytes), and renal tubular necrosis. Clinical pathologies included plasma hypernatraemia and hyperchloridaemia [28,52]. Barramundi held in KDSGW were able to stabilise the circulating level of K^+ by withdrawing K^+ from muscle tissue. This flux was unsustainable and eventually led to death; effectively a diagnosis of hypokalaemic myopathy [28,52]. Whether or not similar pathology is occurring in snapper transferred to KDSGW is yet to be elucidated. Barramundi, being a catadromous species would be expected to have both freshwater and seawater ionocytes and the interplay between these ionoregulatory mechanisms might mean their response and adaptability to K^+ deficient water bodies is different to that of snapper. Snapper are known to vary the number and size of their seawater ionocytes in response to changing salinity [30], however this adaptive change to salinity does not appear to improve their ability to adapt to KDSGW, even though the overall concentration of other external ions (e.g., salinity) in KDSGW was fairly normal.

With respect to the external composition of the rearing medium, it appears the efflux of K^+ from fish is influenced more by the external [Na^+] than the external [K^+]. An older study on K^+ flux in rainbow trout and sculpin reared in K^+ deficient seawater indicated that the efflux of K^+ decreased significantly and the trans-epithelial potential (TEP) decreased slightly when K^+ was absent from the medium [12]. These authors also showed that the [K^+] of the external medium did not overly influence efflux of K^+ from the gills, however K^+ efflux was sensitive to the [Na^+] and possibly the osmotic pressure and ionic strength of the external medium. This was supported by experiments on the effect of external [K^+] on the TEP and rate of Na^+ efflux in fat sleeper *Dormitator maculatus*, which showed that Na^+ efflux was greatly reduced when the fish were transferred to K^+ free seawater [53]. Reduced Na^+ efflux may partly explain why the juvenile snapper did not survive in KDSGW and why they failed to thrive in KDSGW fortified to 40% [K^+] of equivalent salinity seawater; the lower critical limit for [K^+] established by Fielder et al. [5]. It should be noted that Fielder et al. [5] demonstrated that it was the addition of K^+ as KCl not Cl^- as NaCl which increased survival of juvenile snapper in KDSGW. The [Ca^{2+}] of KDSGW was 150% higher than estuarine water of equivalent salinity. This may

have implications for our study as the ionic permeability of the gill in many species is affected by [Ca^{2+}] in the water [32]. Nonetheless, Partridge and Lymbery [52] found no evidence that elevated levels of magnesium (e.g., 187% [Mg^{2+}] of equivalent salinity seawater) affected the performance of Barramundi reared in KDSGW.

The multifactor approach adopted for Exp. 2 demonstrated that it was the direct fortification of KDSGW with KCl, not the fortification of the aquafeed, which was responsible for ameliorating the low [K^+] of KDSGW. This was exemplified by the fact that there was an approximately 59% and 79% improvement, respectively, in the SGR and FCR of fish reared in either estuarine water (control) or the fully fortified KDSGW treatment, compared to fish reared in KDSGW adjusted to 40% [K^+] of equivalent salinity estuarine water, irrespective of the amount of KCl added to the aquafeed (Table 5). As expected, fortification of KDSGW to 40% [K^+] of equivalent salinity estuarine water (i.e., 90 mg K^+ L^{-1}) prevented mortality [5], but this level of fortification was not sufficient to promote optimal growth and feed utilisation; presumably due to the additional energy costs associated with either osmotic, ionic, or acid base regulation [54]. That is, the aforementioned energy debts effectively limited the amount of metabolic energy available for somatic growth [55]. The fact that snapper responded both rapidly and positively to increases in the [K^+] of the rearing medium and not the diet suggests that uptake of K^+ from the diet was not occurring or that other mechanisms involved in maintaining hydromineral balance were compromised.

The role the GI tract plays in terms of water balance is well documented, however its role in absorption of critical electrolytes is less understood [20,21]. The absorption of salts and critical ions from aquafeed is likely to vary considerably depending on the composition of the water that the fish are drinking and whether it inhabits a freshwater or marine environment. Recent studies have suggested that it is the level of Cl^-, not Na^+, that triggers the external drinking response in saltwater teleosts and the negative feedback system of the internal salt concentrations in the lumen (other authors cited in [20]). Ingestion of dry commercial aquafeed will also alter the drinking rate and may change the osmotic pressure in different sections of the GI tract. Ion uptake from the GI tract is thought to be more critical in freshwater fish than marine fish, thus the interplay and balance of other electrolytes, especially Na^+ and Cl^- with K^+, and the impact this has on water absorption from the GI tract, are inextricably linked.

4. Materials and Methods

4.1. Animal Care and Ethics Statement

This study was performed under the NSW DPI Fisheries Animal Care & Ethics (ACEC) Research Authority known as the 'Aquaculture Nutrition ACEC 93/5–Port Stephens' (2004). Care, husbandry, and termination of fish were carried out according to methods outlined in 'A Guide to Acceptable Procedures and Practices for Aquaculture and Fisheries Research' [56].

4.2. Overview of Experimental Design

Two experiments were carried out to evaluate the efficacy of fortifying a commercial aquafeed with various levels of KCl. The fortified feeds were fed to juvenile snapper reared in KDSGW or suitable control treatments. Exp. 1 investigated if short term acclimation of juvenile snapper to diets fortified with 0 (residual), 25, or 50 g KCl kg^{-1} affected survival, feed intake, and specific growth rate (SGR) of fish transferred immediately from estuarine water into unfortified KDSGW of equivalent salinity. Exp. 2 investigated whether feeding diets fortified with 0 (residual), 10, or 25 g KCl kg^{-1} affected survival, feed intake, and SGR of fish reared in estuarine water for 13 days, or affected the same metrics when fish were subsequently transferred into fortified KDSGW; in this case KDSGW was fortified to 40% or 100% the [K^+] of equivalent salinity estuarine water. Saltwater used in both experiments was sourced from an estuary adjacent to the NSW DPI Port Stephens Fisheries Institute (PSFI).

4.3. Feed Preparation

The same batch of aquafeed (Ridley Agriproducts Pty. Ltd., Narangba, QLD, Australia; 50% crude protein; 12% crude fat; 18.0 MJ·kg^{-1} gross energy) was finely ground through a laboratory scale hammer mill fitted with a 1.5 mm screen (Raymond Laboratory Mill, Transfield Technologies, Rydalmere, NSW, Australia). Four diets were prepared by adding 0, 10, 25, or 50 g KCl kg^{-1} of mash (KCl was 99.5% analytical reagent grade; Chem-Supply Pty. Ltd., Gillman, SA, Australia) and dry mixing for a minimum of 15 min before addition of a suitable quantity of distilled water (Hobart Mixer: Troy Pty Ltd., Troy, OH, USA). Sinking pellets were formed by passing the dough through a meat mincer fitted with a 2.0 mm die (Barnco Australia Pty Ltd., Leichhardt, NSW, Australia). Moist pellets were dried (≈35 °C) until the moisture content was <100 g·kg^{-1}. Following preparation, all diets were stored frozen at <−15 °C until required. Proximate and mineral compositions of experimental diets are presented in Table 1. Chemical analysis of the diets and ingredients were performed exclusively by NATA accredited Food & Agricultural Laboratories of Australia Pty. Ltd. (FALA; Coopers Plains, QLD, Australia, http://www.fala.com.au/).

4.4. Water Management, Preparation, and Chemistry

A single volume of hypersaline KDSGW (75–80 g·L^{-1}) was transported from the NSW DPI Inland Saline Aquaculture Research Institute at Wakool, NSW to PSFI and stored in a covered fibreglass holding tank until required. To allow comparisons with previous research, water treatments in this study were adjusted to a salinity of 20 g·L^{-1} using rainwater obtained from a single source at PSFI (i.e., salinity < 3.3 g·L^{-1}; [K$^+$] < 1.5 mg·L^{-1}). Prior to adjusting the salinity, the [K$^+$] of stored volumes of KDSGW, estuarine water, and rainwater were determined in order to accurately fortify experimental volumes (200 L batches) of diluted KDSGW groundwater with KCl. The water chemistry of typical seawater [14] as well as the chemistry of full strength and diluted volumes of estuarine and KDSGW are presented in Table 2. Water chemistry analysis of pH, salinity, and K$^+$ was conducted by Hunter Water Laboratories (Warabrook, NSW, Australia; http://www.hwa.com.au/), whereas the concentration of other major and minor ions was estimated according to dilution ratios.

4.5. Experimental Facility and General Procedures

The snapper used in this study were the progeny of first generation brood-stock held at PSFI. Groups of fish were anaesthetised prior to handling (Benzocaine solution; 10–15 mg·L^{-1}) before individual fish were selected, lightly dried with a damp absorbent cloth, weighed, and placed into experiment tanks. In this way, Exp. 1 and Exp. 2 were stocked with 8 and 6 fish per tank, respectively. Fish were also individually weighed during and at the completion of each experiment.

Experimental units were housed in a photoperiod controlled laboratory (12L:12D; fluorescent lighting). Each unit consisted of a rectangular 60 L clear acrylic aquarium connected to a 9 L plastic sump by 20 mm plastic tubing. Each sump contained approximately 2 L of bio-media ("B-Cell"; Water Management Technologies Inc., Baton Rouge, LA, USA). A small fountain pump (Watermaster, White International, Alexandria, NSW, Australia) was housed within the plastic sump to re-circulate the water in each unit. Water was pumped from the sump (≈1.6 L·min^{-1}) into the aquarium before returning to the sump via gravity flow through a simple particle trap constructed from a perforated plastic container (200 mL) filled with filter wool. Each aquarium and associated sump was aerated using fine-bubble air-stone diffusers. The filter wool from each particle trap was removed each day, washed in freshwater (salinity < 0.3 g·L^{-1}) and replaced. In order to maintain the water quality of individual units and to control the build-up of organic matter, 3–5 L·day^{-1} of water was siphoned from each aquarium and replaced with clean water of the desired water chemistry.

Water quality variables were recorded from each experiment tank using one of two hand held water quality analysers; either a Model 611 (Yeo-Kal Electronics, Brookvale, NSW, Australia) or a Horiba U-10 (Horiba, Japan). Total ammonia [NH_3 + NH_4^+] was monitored from tanks using a rapid test

kit procedure (Model 1.08024.0001, E. Merck, Darmstadt, Germany). During Exp. 1, the temperature, dissolved oxygen, salinity, and pH ranged from 20.3–26.0 °C, 4.9–8.2 mg·L^{-1}, 20.1–28.9 g·L^{-1}, and 7.0–8.3 units, respectively, and [NH_3 + NH_4^+] was $\leq$ 1.0 mg·L^{-1}. During Exp. 2 the temperature, dissolved oxygen, salinity, and pH ranged from 21.8–26.0 °C, 4.9–7.0 mg·L^{-1}, 19.6–32.3 g·L^{-1}, and 6.0–8.0 units, respectively, and [NH_3 + NH_4^+] was $\leq$ 0.7 mg·L^{-1}.

4.6. Exp. 1: Effect of KCL Fortified Aquafeed on Feed Intake and Direct Transfer of Fish to K^+ Deficient Saline Groundwater (KDSGW)

Each of the 3 test diets (i.e., residual, 25, or 50 g KCL kg^{-1}) were randomly allocated to 6 experimental units (n = 6) and each tank of fish was hand-fed to apparent satiation twice daily (i.e., 0830 h and 1500 h). For the first 6 days fish were held in normal estuarine water (salinity = 23.5 g·L^{-1}) while they were fed the experimental diets. During this period, the salinity of the estuarine water in all tanks was gradually reduced to 20 g·L^{-1} by using stocks of pre-diluted estuarine water to replace water that was siphoned off during tank cleaning procedures. On day 7, all fish were introduced into their respective water treatments following their usual morning meal (≈1000 h). The procedure involved rapidly draining all but 5 L of the estuarine water from each aquarium (note that sumps were also completely drained), before refilling aquaria with stocks of KDSGW or estuarine water (control) that had been diluted to a salinity of 20 g·L^{-1} and adjusted to a similar water temperature. After water transfer, flows were restored in each of the experimental units and feed was offered according to the aforementioned protocol.

4.7. Exp. 2: Effect of KCl Fortified Aquafeed on Transfer of Fish to Fortified K^+ Deficient Saline Groundwater (KDSGW)

This experiment was conducted in 2 stages. In the first stage, the fish were reared for 13 days in estuarine water (salinity = 31 g·L^{-1}) and fed diets fortified with residual, 10, or 25 g KCl·kg^{-1} to determine if the concentration of KCl affected short term feed intake and SGR (n = 9). In the second stage, 3 of the tanks assigned to each diet group were randomly allocated to 1 of 3 water treatments: (1) KDSGW fortified to 40% [K^+] of equivalent salinity estuarine water; (2) KDSGW fortified to 100% [K^+] of equivalent salinity estuarine water; (3) estuarine water (control). The salinity of all water treatments in stage 2 was 20 g·L^{-1}. The transfer of the fish from stage 1 to stage 2 occurred on day 13 when the fish were removed from the experiment tanks for bulk weighing. At this time, all aquaria were thoroughly washed, rinsed clean, and filled with the desired water treatment before the fish were returned. The experiment was run for a further 21 days.

4.8. Statistical Analysis

Exp. 1 was designed for analysis using two-way ANOVA with the water treatment and the level of KCl as fixed factors. Exp. 2 was designed for analysis using one-way (stage 1) or two-way ANCOVA (stage 2). In the case of ANCOVA, the interim weight of fish was used as the covariate to control for minor differences in the weight of snapper at the end of stage 1. Prior to conducting ANOVA, data were tested to ensure that treatment variances were homogenous (Levene's test). Where treatment variances were heterogeneous, data were log transformed. The significance level for all ANOVA and multiple comparisons tests (Tukey-Kramer post-hoc test) was set at 0.05 and data were statistically analysed using NCSS Version 8.0.23 (NCSS, LLC., Kaysville, UT, USA; www.ncss.com).

5. Conclusions

The results from our study reconfirm juvenile snapper can survive in KDSGW that has been adequately fortified with KCl. However, improvements in SGR and FCR were recorded when KDSGW was fortified to contain 100% [K^+] of equivalent salinity estuarine water. High survival rates of fish reared in fortified KDSGW as well as improvements in their SGR and FCR suggest that the imbalance of ions in KDSGW, such as Ca^{2+}, compared to estuarine water were not detrimental to the fish and

were therefore not problematic in terms of snapper maintaining hydromineral homeostasis. We feel it is unlikely that the level or balance of other dietary minerals in the aquafeed we used were deficient, however this presumption is based on general estimates for the mineral requirements of fish, many of which are based on freshwater studies [51,57]. Fortification of a commercial aquafeed with up to 5% KCl was not beneficial in terms of improving the survival of juvenile snapper transferred directly into KDSGW. Similarly, fortification of an aquafeed with up to 2.5% KCl was not beneficial in terms of improving the growth rate and FCR of juvenile snapper reared in KDSGW fortified to 40% [K^+] of equivalent salinity seawater. Rearing snapper in KDSGW such as that from the MDB will remain reliant on fortifying these water sources with a suitable level of potassium salts.

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