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RESEARCH ARTICLE | *Hormones, Reproduction and Development*

Evidence for a role of arginine vasotocin receptors in the gill during salinity acclimation by a euryhaline teleost fish

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Lema SC, Washburn EH, Crowley ME, Carvalho PG, Egelston JN, McCormick SD. Evidence for a role of arginine vasotocin receptors in the gill during salinity acclimation by a euryhaline teleost fish. *Am J Physiol Regul Integr Comp Physiol* 316: R735–R750, 2019. First published March 27, 2019; doi:10.1152/ajpregu.00328.2018.—The nonapeptide arginine vasotocin (AVT) regulates osmotic balance in teleost fishes, but its mechanisms of action are not fully understood. Recently, it was discovered that nonapeptide receptors in teleost fishes are differentiated into two V1a-type, several V2-type, and two isotocin (IT) receptors, but it remains unclear which receptors mediate AVT's effects on gill osmoregulation. Here, we examined the role of nonapeptide receptors in the gill of the euryhaline Amargosa pupfish (*Cyprinodon nevadensis amargosae*) during osmotic acclimation. Transcripts for the teleost V1a-type receptor *v1a2* were upregulated over fourfold in gill 24 h after transferring pupfish from 7.5 ppt to seawater (35 ppt) or hypersaline (55 ppt) conditions and downregulated after transfer to freshwater (0.3 ppt). Gill transcripts for the nonapeptide degradation enzyme leucyl-cystinyl aminopeptidase (LNPEP) also increased in fish acclimating to 35 ppt. To test whether the effects of AVT on the gill might be mediated by a V1a-type receptor, we administered AVT or a V1-type receptor antagonist (Manning compound) intraperitoneally to pupfish before transfer to 0.4 ppt or 35 ppt. Pupfish transferred to 35 ppt exhibited elevated gill mRNA abundance for cystic fibrosis transmembrane conductance regulator (*cftr*), but that upregulation diminished under V1-receptor inhibition. AVT inhibited the increase in gill Na^+/Cl^- cotransporter 2 (*ncc2*) transcript abundance that occurs following transfer to hypoosmotic environments, whereas V1-type receptor antagonism increased *ncc2* mRNAs even without a change in salinity. These findings indicate that AVT acts via a V1-type receptor to regulate gill Cl^- transport by inhibiting Cl^- uptake and facilitating Cl^- secretion during seawater acclimation.

isotocin; ion transport; LNPEP; nonapeptide; pupfish; vasopressin

INTRODUCTION

Teleost fishes cope with changes in environmental salinity by modulating water and ion exchange across several tissues to maintain hydromineral balance (54). Hormones play a critical role in regulating dynamics of water and ion exchange by coordinating drinking behavior, ion transporter activity and recruitment, and water and ion excretion under shifting osmotic stresses (65, 90). These hormonal effects on osmotic balance can occur gradually, as hormones up- or downregulate gene expression and or promote cell proliferation and recruitment, or can occur much more rapidly via changes in intracellular cAMP or Ca^{2+} concentrations, protein phosphorylation, or membrane recruitment of ion or water channels. Ultimately, the time scale of endocrine mediation of osmotic regulation depends on the tissue, the type of hormone, and the mechanism of hormone action.

The mammalian neurohypophysial peptide hormone arginine vasopressin (AVP) has long been implicated in regulating osmotic balance across mammals via rapid action on tissues such as the kidney (9, 77). AVP is one mammalian form of the nonapeptide family of AVP/arginine vasotocin (AVT) and oxytocin (OT)/mesotocin (MT)/isotocin (IT) neurohypophysial hormones of vertebrates (1, 8, 21, 22, 32, 67, 78, 96, 97). These vertebrate peptides belong to a larger family of evolutionarily homologous, metazoan nonapeptides that share a common nine-amino acid structure that includes a disulfide bridge linking cystine residues in positions 1 and 6 (e.g., 18, 82, 91, 104). In mammals, it is well established that AVP regulates renal water retention via action on V2-type nonapeptide receptors and aquaporin-2 channels (72, 79, 101). Just as AVP functions in the maintenance of osmotic balance in mammals, several studies provide evidence that neurohypophysial nonapeptides have an osmoregulatory role in nonmammalian vertebrates (2, 52, 80, 89, 95).

Some of the clearest evidence for nonapeptides regulating hydromineral balance in nonmammalian vertebrates comes from studies of teleost fishes. In fish, plasma AVT concentrations correlate positively with plasma osmolality under varying osmotic conditions, suggesting a role for AVT in maintaining salt and water balance (7, 11). AVT mRNA levels in the hypothalamus have been found to respond to changes in environmental salinity in several fishes (e.g., 27, 45, 52), and these mRNA changes appear to result in altered hypothalamic AVT peptide abundance. Amargosa pupfish (*Cyprinodon nevadensis amargosae*), for instance, raised in a higher-salinity environment (3 ppt) show a reduced number of AVT-immunoreactive (AVT-ir) neurons in the magnocellular region of the hypothalamic preoptic area compared with fish at 0.4 ppt (47, 48), whereas freshwater-acclimated Japanese medaka (*Oryzia latipes*) experience a reduction in AVT-ir magnocellular neuron number when moved to seawater (23). Paralleling these changes in hypothalamic AVT expression, pituitary AVT lev-

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els increase in medaka and flounder (*Platichthys flesus*) transferred from high salinities to freshwater (23, 99), and decrease when freshwater-acclimated medaka and flounder are moved to seawater (23, 103). These salinity-induced alterations to pituitary AVT levels appear related to changes in posterior pituitary AVT storage, as hyperosmotic conditions have been observed to enhance pituitary AVT secretion and increase circulating AVT concentrations, whereas hypoosmotic stresses have the opposite effects (11, 35, 36, 61; reviewed in 6, 7, 37, 102).

AVT appears to affect hydromineral balance in fish by regulating ion and water transport across several osmoregulatory tissues. Early functional evidence for a role of AVT in osmoregulation in fishes came from in vivo studies, where AVT was found to alter glomerular filtration and induce vasopressor effects (5, 24). Renal functions for AVT were subsequently confirmed in situ using perfused kidney tissue of rainbow trout (*Oncorhynchus mykiss*), where AVT induced dose-dependent increases in glomerular filtration and decreases in urine production (3). AVT also regulates ion exchange across the gill epithelium, which is the primary site of ion uptake in freshwater and secretion in seawater. In cultured gill epithelia from European sea bass (*Dicentrarchus labrax*) and mummichog (*Fundulus heteroclitus*), both AVT and IT stimulated Cl^- secretion (20, 58). In sea bass, a V1-type receptor agonist also stimulated Cl^- secretion, whereas V1-type receptor antagonism, but not V2-type receptor antagonism, reversed AVT's effects on Cl^- secretion in cultured gill epithelium (20), which is composed predominantly of respiratory pavement cells but also contains smaller numbers of ionocytes (also referred to as "mitochondria-rich" cells) that function in gill Cl^- transport. Taken as a whole, these findings point to a role for both AVT and IT action on gill Cl^- secretion while also suggesting that a V1-type nonapeptide receptor mediates AVT's effects on the gills.

While mammals have evolved three G-coupled membrane receptors for AVP (V1a-type, V1b-type, and V2-type) and an OT receptor, it was recently discovered that teleost fishes have evolved a distinct diversity of nonapeptide receptors derived from multiple gene duplication events (45, 74, 105). Conserved gene synteny patterns point to a single ancestral nonapeptide receptor having diverged into the AVP/AVT and OT/MT/IT receptor forms during the early vertebrate 2R gene duplication event, followed by the teleost-specific duplication to generate additional nonapeptide receptors in teleost fishes (39). Those duplication events generated two distinct V1a-type receptors (V1a1 and V1a2) and at least three V2-type receptors (V2a, V2b, and V2c), as well as two IT receptors in teleosts (34, 39, 45, 63, 74, 105). Given the expanded diversity of nonapeptide receptors in fishes, it remains unclear which of these receptors mediate the effects of AVT on osmoregulation by the gill.

In this study, we explore the function of nonapeptide receptors in osmoregulation by the gill epithelium of Amargosa pupfish, *C. n. amargosae* (Amargosa River subspecies), a species belonging to a monophyletic group of euryhaline pupfishes that evolved in the arid Death Valley region of California and Nevada within the last ~20,000 years (88). Pupfishes generally show the ability to inhabit environments with widely ranging salinities (25), and taxa belonging to the Death Valley clade have been shown to tolerate environmental salinities from freshwater up to 105 ppt (71). Pupfishes, similarly to other teleost fishes, have evolved two distinct V1a-type receptors, at least five V2-type receptors, and two IT receptors (Fig. 1), several of which had not been reported previously in this taxon (45). Here, we explored the role of nonapeptide receptors in gill osmoregulation in Amargosa pupfish by *1*) examining changes in the relative abundance of mRNAs encoding nine distinct AVT and IT receptors in the gill epithelium of pupfish acclimated to 7.5 ppt (brackish) and then transferred to freshwater (0.3 ppt), seawater (35 ppt), or hypersaline (55 ppt) conditions, and *2*) manipulating the AVT system by treating pupfish with exogenous AVT or a V1-type receptor antagonist before salinity transfer. We thus first examined transcriptional responses of these receptors in the gill of pupfish acclimating to increased or decreased salinities to identify changes in nonapeptide receptor expression during salinity acclimation. This approach provided evidence that the pupfish V1a2 V1-type receptor shows transcriptional changes in the gill in a pattern suggestive of a role in osmoregulation. We therefore performed a second experiment by pharmacologically treating pupfish with either exogenous AVT or a V1 receptor antagonist (Manning compound) to examine whether AVT influences salinity-induced changes in gill ion channel or transporter expression during salinity acclimation. To gain a more comprehensive picture of nonapeptide actions in the gill during salinity acclimation, we also examined transcriptional changes in the leucyl-cystinyl aminopeptidase (LNPEP) enzyme, also known as vasopressinase or oxytocinase, a membrane-bound, zinc-dependent metalloexopeptidase that inactivates nonapeptides of the AVT/IT family as well as selected other cyclic polypeptides (62, 94). A complementary DNA (cDNA) encoding this putative AVT/IT degradation enzyme was recently identified in Amargosa pupfish, and messenger RNAs (mRNAs) for *lnpep* were found to be at relatively high levels in the gill (16). We therefore also examined whether transcripts encoding LNPEP vary in the gill in patterns indicative of expressional regulation by hyper- or hypoosmotic conditions.

MATERIALS AND METHODS

Phylogenetic Analysis of AVT and IT Receptor Diversity in Pupfish

Sequences for the previously identified *C. n. amargosae* AVT V1a-type receptors V1a1 (GenBank accession no. GQ981412) and V1a2 (GQ981413) (45), the V2-type receptors V2a1 (GQ981414) (45) and V2b1 (KJ668598) (43), and the IT receptor ITR1 (GQ981415) (45) were used to BLAST search the genome of *Cyprinodon nevadensis* (GCA_000776015) and identify genes encoding additional AVT receptors V2a2 (JSUU01000639), V2b2 (JSUU01002888), and V2c (JSUU01002888), as well as an additional IT receptor termed ITR2 (JSUU01008219). The deduced amino acid sequences for these receptors were then aligned to AVT and IT receptor sequences identified from another Cyprinodontiform fish (guppy, *Poecilia reticulata*) as well as from selected other fishes and vertebrates by using Clustal X software v.2.0 (41). Phylogenetic analyses were then conducted via the Neighbor-Joining method with a p distance model and partial deletion of gaps using MEGA v.7 software (38). Confidence values for nodes were calculated by bootstrapping (1,000 replicates).

Tissue Distribution Patterns of AVT and IT Receptor Transcript Abundance

Tissue collection. Sexually mature male and female Amargosa pupfish, *C. n. amargosae*, were collected on 27 June 2015 from the Amargosa River near Tecopa, California (N 35°51.275′, W 116° 13.833′) using minnow traps. Fish were transported to holding facilities at California Polytechnic State University, San Luis Obispo (California) and maintained in 38-l tanks under conditions representing the environmental parameters of the Amargosa River on the day of collection (~30°C, 3.5 ppt salinity, pH 8.1). Fish were fed daily a 1:1 mixture of commercial spirulina (Aquatic Eco-Systems, Apopka, FL) and brine shrimp (San Francisco Bay Brand, Newark, CA) flake feeds. After ~5 mo in captivity, pupfish ($n = 3$ for each sex) were netted, euthanized using tricaine methansulfonate (MS222; 300 mg/l in water buffered with 200 mg/l $NaHCO_3$), and the following tissues were dissected, flash-frozen in liquid N_2, and stored at −80°C: brain, pituitary gland, gills, heart, liver, gonads, kidney, gastrointestinal (GI) tract, and muscle (collected from the left side of the caudal peduncle). The brain was subdivided into the telencephalon, hypothalamus, optic tectum, cerebellum, and hindbrain (medulla oblongata) regions before freezing, as described by Elkins et al. (16). The sex of each pupfish was determined by visually assessing gonadal sex. Mean (±SD) body sizes were as follows: females: 43.40 ± 1.68 mm [standard length (SL)], 3.15 ± 0.84 g (body mass); males: 41.13 ± 4.48 mm SL, 2.15 ± 0.82 g (body mass). All procedures were approved by the Animal Care and Use Committee of California Polytechnic State University (Protocol no. 1507).

RNA isolation and quantitative real-time RT-PCR. Total RNA was extracted for all tissues using TRI Reagent (Molecular Research Center) with bromochloropropane as the phase separation reagent. Total RNA was DNaseI treated (TURBO DNA-free Kit, Ambion), quantified (260/280 ratios > 1.96, NanoPhotometer P300; Implen, Westlake Village, CA), and then diluted. Total RNA was reverse transcribed in reactions containing 1,040 ng of total RNA (16 μl of 65 ng/μl), 500 μM dNTPs (Promega), 800 μg of random primers (Promega), 5 U of recombinant RNasin ribonuclease inhibitor (Promega), 192 U of GoScript reverse transcriptase (Promega), and 3.75 mM $MgCl_2$ (Promega) in a 32-μl reaction volume also containing 6.4 μl 5× buffer (Promega) and 0.275 μl of nuclease-free H_2O. Reverse transcription reactions were run under a thermal profile of 25°C for 5 min and 42°C for 1 h, followed by 70°C for 15 min to inactivate the reverse transcriptase. The relative abundance of mRNAs encoding each of the five AVT receptors and two IT receptors in Amargosa pupfish was then determined using quantitative real-time RT-PCR (qRT-PCR) as described below.

Primers for SYBR Green qPCR assays were designed to protein-coding regions of full-length or partial cDNAs for each nonapeptide receptor gene (Table 1). Primers were also designed to *β-actin* (EU886377) from Amargosa pupfish for use as an internal reference gene (43, 46). All primers were synthesized by Integrated DNA Technologies (Coralville, IA), and the specificity of each SYBR Green primer set was confirmed by Sanger sequencing PCR products.

All qPCR assays were conducted as 16-μl reactions. Each reaction contained 4.5 μl of nuclease-free water (Sigma, St. Louis, MO), 8 μl of iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA), 1 μl each of forward and reverse primers (5 μM), and 1.5 μl of reverse-transcribed cDNA template. The PCR thermal profile for each reaction was 50°C for 2 min, 95°C for 10 min, 42 cycles of 95°C for 15 s, and 59°C for 1 min, and all assays were run on a 7300 Real-Time PCR System (Applied Biosystems). DNA contamination was assessed for each gene by analyzing RNA samples that were not reverse transcribed, and each qPCR run included two samples without cDNA template to further control for contamination. For each gene, a standard curve was made from a pool of RNA from samples representing all treatments and both sexes. This pooled sample was serially diluted and each standard concentration assayed in triplicate. Correlation coefficients (r^2) for the standard curves were always greater than $r^2 = 0.97$. Melt curve analyses was also performed to confirm amplification of a single product and the absence of primer-dimers during each quantitative PCR run. PCR efficiencies for each gene were calculated using the equation: % efficiency = $[10^{(-1/\text{slope})} - 1] \times 100$. Mean efficiencies for each qPCR assay are provided in Table 1. For each gene, relative mRNA levels were normalized to mRNA abundance values for *β-actin* in each tissue, and abundance values of each gene of interest were then expressed as a relative level by

Table 1. *Nucleotide sequences of primers for SYBR Green quantitative PCR in Amargosa pupfish (C. nevadensis amargosae)*

Transcript	Primer	Nucleotide Sequence (5′ to 3′)	Amplicon Length (bp)	% Efficiency (Mean)
v1a1	Forward	CGA TCT GGA GGA ATC TGA AGT G	144	101.81
	Reverse	TCA TCT TGA CCG TGC GTA AT		
v1a2	Forward	TTA CTG ACC AGG ATC ACC AAT C	111	100.26
	Reverse	GGA GCT ACT GCC TGT TTA CTC		
v2a1	Forward	GGG ACA TAA CAG AGA GGT TTC AG	111	99.15
	Reverse	CGA CTG TCA TGG CAA CTA TCA		
v2a2	Forward	GGA TGT TCG CCT CCT CTT AC	124	101.26
	Reverse	GCC AGA AAT ATG AAA GTG TTC C		
v2b1	Forward	CCC TGC ATC TAC CTG CTA TTC	98	98.47
	Reverse	TCG TGC ATT GGG TCC TTA C		
v2b2	Forward	CCA GTA ACA CCA GTT TGG AGA G	89	101.14
	Reverse	GAT GGA CAG CAG GGC TAT TT		
v2c	Forward	CCG CAG CTC TCC ATA GAA ATT A	105	99.52
	Reverse	CAT ATA GGC AGA GGC GAA CAT C		
itr1	Forward	CAC CTG TTC CAT GAC CTG ATA A	90	101.27
	Reverse	CCT GCA CTC CTG ATT GAC AT		
itr2	Forward	CCG ATC TGG TCG TTG CTA TAT T	105	100.04
	Reverse	GCA GGT ATT TCA CCA ACC TAC A		
lnpep	Forward	ATG CTG AGA AGC GGA AGA TAC	113	102.28
	Reverse	CAG TTC CTG TGT CTG GAT GAT AG		
ef-1α	Forward	CCT GGG TAT TGG ACA AAC TGA	90	101.72
	Reverse	CGT AGT ACT TGC TGG TCT CAA A		
β-actin	Forward	CTT CCT TCC TTG GTA TGG AGT C	93	100.54
	Reverse	CAT ACA GGT CCT TAC GGA TGT C		

bp, base pair; *itr*, isotocin receptor; *lnpep*, leucyl-cystinyl aminopeptidase; *ef-1α*, elongation factor 1α.

dividing resulting values by the mean expression level of that gene in the forebrain tissue of male pupfish. Results showing the distribution of *lnpep* mRNAs in these same fish are provided in Elkins et al. (16).

Role of AVT and IT Receptors in Salinity Acclimation

Animals. Amargosa pupfish were collected from the Amargosa River (N 35°51.275′, W 116°13.833′) on 18 November 2012 using minnow traps. Salinity of the Amargosa River on that collection day was recorded at 7.5 ppt (YSI 85, YSI, Yellow Springs, OH); for that reason, a salinity of 7.5 ppt was selected as the "control" salinity for the experiments described below. After collection, pupfish were transported to California Polytechnic State University, San Luis Obispo, California, where they were maintained in 208-liter closed-system tanks in 7.5 ppt water made with Instant Ocean salt (Unified Pet Group, Blacksburg, VA) and deioinized water. Fish were maintained on a 14:10-h light-dark photoperiod at 24–25°C for the duration of the experiments, and fed ad libitum daily a mixture of spirulina and brine shrimp flake feeds as described above.

Experiment 1: salinity acclimation challenge. In the first experiment, adult pupfish acclimated to 7.5 ppt were transitioned to lower (0.3 ppt) or higher (35 or 55 ppt) salinities over a 4-h period. Fish were then maintained under these altered salinities for 14 days to evaluate the effects of salinity acclimation on gill nonapeptide receptor mRNA levels. Additional fish were maintained at 7.5 ppt as a control. Salinity conditions in each tank were recorded daily (YSI model 85, Dissolved Oxygen and Conductivity Meter; YSI) and tank temperatures (26.68 ± 1.26°C, means ± SD; no differences among treatment groups) were recorded every 30 min (HOBO U12 External Data Loggers; Onset, Bourne, MA). Fish were fed throughout the experiment, including on the day of the salinity change and all 14 days thereafter. All physiological measurements in these fish are reported relative to the start time (0 h, baseline) for beginning the 4-h salinity change. Only data for the responses of nonapeptide receptor mRNA levels in the gill epithelium are provided here; data for how these salinity treatments altered plasma osmolality, gill Na^+-K^+-ATPase (NKA) activity, and relative mRNA levels of several ion channels, ion cotransporters, and aquaporins in the gills of these pupfish are described elsewhere (42). Additional details of the methods for this salinity acclimation experiment are also provided in Lema et al. (42).

In the hour immediately before commencement of the salinity challenge, one fish from each experimental tank was netted and quickly euthanized in tricaine methanesulfonate (MS222) to provide an initial baseline sample. Fish were measured and weighed, the caudal peduncle was severed, blood was collected in heparinized hematocrit tubes, and gill filaments were dissected and divided between two samples: one sample of gill filament was flash-frozen in liquid N_2 for mRNA expression analyses using qRT-PCR, while the other sample was preserved for Na^+-K^+-ATPase (*NKA*) activity measurement. After collection of this baseline (*day 0*) sample, salinity conditions in each tank were changed over a 3- to 4-h period to create the following treatments: 0.3 ppt (freshwater; 18 mosmol/kg H_2O, pH 7.64), 35 ppt (seawater; 1,060 mosmol/kg H_2O, pH 8.19), 55 ppt (hypersaline; 1,730 mosmol/kg H_2O, pH 8.31), or alternatively maintained unchanged at 7.5 ppt (control group; 216 mosmol/kg H_2O, pH 7.68). Eight replicate tanks were used for each treatment group. Salinity conditions of each tank were changed by pumping different salinity water (Instant Ocean, Unified Pet Group) into the biological filter sump for each closed-system tank. This approach meant that pupfish experienced a change in salinity conditions within their experimental 38-liter tanks without any need for netting and transferring fish between tanks, thereby avoiding any induction of a physiological stress response from handling, which might also alter patterns of gill nonapeptide receptor expression via mechanisms independent of salinity. One individual fish was then sampled from each of the eight replicate tanks ($n = 8$) at time periods of 8, 24, and 96 h and 14 days after transitioning fish to different salinities.

At all sampling times, each fish sampled was euthanized using MS222 and then measured and weighed [females: 34.8 ± 0.05 mm standard length (SL), 1.41 ± 0.06 g mass; males: 39.8 ± 0.6 mm SL, 2.37 ± 0.10 g mass; means ± SE)]. Blood was transferred to heparinized microcentrifuge tubes and centrifuged at 3,000 *g* for 15 min at 4°C. The resulting plasma was collected and stored at −80°C. The first, second, and third gill arches on the right side of each fish were dissected and immediately frozen in liquid N_2 for gene transcript analyses using qRT-PCR. Filament tissue from the first, second, and third gill arches on the left side of each fish was isolated and preserved in a sucrose-Na_2EDTA-imidazole (SEI) buffer for quantification of NKA activity. Gill tissue for RNA extraction and the gill samples for NKA activity were stored at −80°C until analyzed (for additional details, see Ref. 42).

The relative abundance of AVT and IT receptors in the gill was measured using qRT-PCR as described above. Primers were also designed to a full-length cDNA encoding the LNPEP nonapeptide degradation enzyme (*lnpep*) from *C. n. amargosae* (KY290248) (16) and also to a partial cDNA encoding elongation factor-1α (*ef1α*, EU906930) for use as an additional internal control gene (43, 46). Mean efficiencies for each qPCR assay are provided in Table 1. For each gene, relative mRNA levels were normalized to the geometric mean of mRNA abundance values for the reference genes *ef-1α* and *β-actin*, which did not vary among treatments. Abundance values of each gene of interest were then expressed as a relative level by dividing the resulting values by the mean value of control treatment group.

Experiment 2: effects of AVT or a V1-type receptor antagonist on gill osmoregulation. Pupfish acclimated to 7.5 ppt for over 6 mo were assigned haphazardly to 38-liter tanks with four fish per tank and maintained in those conditions for 14 days. Fish were collected by netting, anesthetized with MS222, and weighed (±0.01 g). Fish were then injected intraperitoneally (5.0 μl Hamilton syringe, ± 0.05 μl) with either a saline solution control [1.0% NaCl with 0.2% bovine serum albumin (BSA)], 1 μg AVT/g body mass in saline solution, or 3.5 μg of the mammalian V1-type receptor antagonist Manning compound [(β-mercapto-β,β-cyclopentamethylenepropionyl^1,*O*-me-Tyr^2,Arg^8)-vasopressin; Sigma-Aldrich, St. Louis, MO] per gram body mass in saline solution. Manning compound has been shown to be a potent in vivo V1a receptor antagonist in mammals (53). Treatment doses were selected based on other peptide injection studies in fishes, specifically prior studies demonstrating AVT effects on osmoregulatory responses in sea bream (82) and behavioral responses in pupfish (49). The concentration of Manning compound was similarly selected based on the range of intraperitoneal injection doses shown previously for these chemicals to alter social behaviors in Amargosa pupfish (49). Immediately after recovery from anesthetization, fish were transferred to a new 38-liter tank at one of following salinities: 7.5 ppt (salinity control), 0.4 ppt, or 35 ppt.

At 24 h after injection and transfer, fish were then netted, euthanized (MS222), weighed, and measured. That 24-h time was selected to correspond to the peak change in *v1a2* mRNAs observed during salinity acclimation from *experiment 1* above. Blood was collected by severing the tail, and gill tissues were dissected and preserved for qRT-PCR or NKA activity analyses. Blood was centrifuged at 3,000 *g* for 15 min at 4°C, and the resulting plasma was stored at −80°C for osmolality quantification.

Plasma osmolality measurement. Osmolality (mosmol/kg H_2O) was quantified from a 5-μl plasma volume using a Wescor 5500 Vapor Pressure Osmometer (Wescor, Logan, UT). Water samples from each recirculating tank system were also collected similarly to blood samples and assayed for osmolality. Water samples were measured in triplicate, and plasma samples were run in duplicate when plasma volume allowed. Osmolality measurements had a coefficient of variation (CV) of 0.99%.

Gill NKA activity. Gill NKA activity was determined using a temperature-regulated microplate protocol (64). Gill filaments were

homogenized in SEI buffer with 0.1% sodium deoxycholate (150 μl) and pelleted by centrifugation. Ouabain-sensitive NKA activity was then quantified via ADP production and nicotinamide adenine dinucleotide (NADH) oxidation in the presence or absence of 0.5 mM ouabain, which acts as a NKA inhibitor. Samples were measured at 340 nm for 10 min at 25°C using a THERMOmax microplate reader (Molecular Devices, Menlo Park, CA), and NKA activity was determined as the difference between the NADH decay slope with and without ouabain and then normalized to the sample's respective total protein concentration. All samples were run in duplicate. NKA values exhibited an intra-assay %CV of 6.5% and interassay %CV of 11.3%. Protein concentrations for each homogenate were measured using a Pierce BCA protein assay.

Quantitative real-time RT-PCR. As described above, total RNA was extracted from gill filaments using Tri Reagent (Molecular Research Center) and then treated with DNase I (TURBO DNA-free kit, Ambion). Reverse transcription reactions were conducted in 22-μl reaction volumes with the same relative volumes and concentrations of reagents as described above. Primers for SYBR Green quantitative real-time PCR assays were designed to protein coding regions of cDNAs for selected ion transporters, ion channels, and aquaporins identified previously from *C. n. amargosae* (42). These particular ion transporters and channels were selected because transcripts encoding these proteins were previously observed to change expression in the gill of pupfish during acclimation to higher or lower salinities (42). Quantitative real-time PCR assays were conducted as described above. Mean PCR efficiencies for gene encoding ion channels, cotransporters, or aquaporins ranged from 98.15 to 103.99%, similar to the efficiencies for these same primer sets provided in a previous study (42). For each gene, relative mRNA levels were normalized to the geometric mean of mRNA abundance values for the reference genes *ef-1α* and *β-actin*, which did not vary among treatments, and were expressed as a level relative to the mean values of the control treatment.

Statistical analyses. Because the actions of nonapeptide hormones including AVT and expression of nonapeptide receptors in selected tissues have been shown to be sexually dimorphic for fish behavior (e.g., Ref. 45), we first tested for any effects of sex on relative mRNA levels. No effects of sex were found, so "sex" was not used as a term in any subsequent statistical models.

One-factor ANOVA models were used to compare relative nonapeptide receptor and *lnpep* mRNA levels in the gill at the baseline (0 h) sampling time to confirm that all treatment groups had similar mRNA levels before experiencing the change in salinity. Relative gene transcript abundance for each nonapeptide receptor gene and the *lnpep* gene was then compared among salinity treatments and sampling times by using two-factor ANOVA models with "salinity," "time," and the interaction between these factors. Tukey HSD tests were subsequently used to identify differences between treatments at each time point for any transcripts exhibiting variation in mRNA levels with salinity or time.

For the AVT system manipulation experiment, plasma osmolality and gill NKA activity were each compared using two-factor ANOVA models with "injection treatment," "salinity," and the interaction term for these two main effect factors. Tukey honestly significant difference (HSD) tests were then used to test for effects of AVT system modulation (i.e., injection of AVT, Manning compound, or saline control) on the transcriptional responses of various ion channels or transporters in the gill. For genes where the data failed to conform to parametric assumptions, data were $\log_{10}(x+1)$ transformed before analysis. All statistical tests were two tailed, with $\alpha = 0.05$ and calculated using JMP Pro 13.2.0 software (SAS Institute, Cary, NC).

RESULTS

Phylogenetic Analysis and Tissue Distribution of AVT and IT Receptors

A phylogenetic tree derived from deduced amino acid sequences shows the relationships of the seven AVT receptors and two IT receptors of pupfish relative to selected nonapeptide receptors from other vertebrates including mammals (Fig. 1). Phylogenetic relationships among those deduced receptors illustrate how the diversity of nonapeptide receptors in pupfish group into two V1a-type receptors (V1a1 and V1a2), five V2-type receptors (V2a1, V2a2, V2b1, V2b2, and V2c), and two IT receptors (ITR1 and ITR2).

Gene transcripts encoding all nine pupfish nonapeptide receptors were detected in the pupfish gill epithelium, but individual receptor genes varied in relative mRNA expression in the gill and in other tissues (Fig. 2). Transcripts encoding the *v1a1* receptor were detected in all tissues but were at higher abundance in the forebrain, hypothalamus, and pituitary gland (Fig. 2*A*); *v1a1* transcripts also showed high abundance in the testis despite very low mRNA levels in the ovary, a pattern of sexual dimorphic expression observed previously in fish (44, 45). Transcripts encoding the V1a-type *v1a2* receptor were similarly ubiquitous in all tissues but at highest abundance in the forebrain and hypothalamus (Fig. 2*B*). Transcripts encoding the V2-type receptor *v2a1* were most abundant in the pituitary gland and heart (Fig. 2*C*), and *v2a2* transcripts were most abundant in the hypothalamus, optic tectum, heart, and testis (Fig. 2*D*). Transcripts for *v2b1* and *v2b2* were both at greatest relative abundance in the brain and heart (Fig. 2, *E* and *F*), whereas *v2c* mRNAs showed sexual dimorphism with the highest expression in the testis, and moderate expression in the brain of both males and females (Fig. 2*G*). Transcript levels for the *v2a2, v2b1, v2b2*, and *v2c* AVT receptors were all below detection limits in the pituitary gland. Transcripts encoding the IT receptors *itr1* and *itr2* were at high relative abundance in the forebrain, hypothalamus, and optic tectum regions of the brain (Fig. 2, *H* and *I*). Notably, transcripts encoding *itr2* showed sexual dimorphism in the pituitary gland; all three male pupfish examined showed the highest relative expression levels of *itr2* in the pituitary gland, while *itr2* abundance was below detection limits in the pituitary gland of all females examined (Fig. 2*I*). To our knowledge such sexual dimorphism in *itr2* mRNA levels in the pituitary gland has not previously been documented.

Salinity-Induced Changes in Gill Nonapeptide Receptor mRNA Abundance

Gene transcripts encoding the vasotocin *v1a1* V1a-type receptor showed only minor variation in relative abundance in response to salinity (Fig. 3*A*; $F_{12,140} = 2.12$, $P = 0.019$), with *v1a1* mRNAs in the gill increasing less than twofold at 1 day after transitioning pupfish to 55 ppt. In contrast, transcript abundance for the *v1a2* receptor varied in patterns dependent on time since the salinity transition (Fig. 3*B*; $F_{12,140} = 6.778$, $P < 0.0001$). Pupfish transferred to 55 ppt, and, to a lesser extent, to 35 ppt, exhibited *v1a2* mRNA levels nearly fourfold higher in relative expression 8 h after salinity change but returned to control (7.5 ppt) levels by 4 days after exposure to high salinity. Transcripts encoding *v1a2* also were lower in

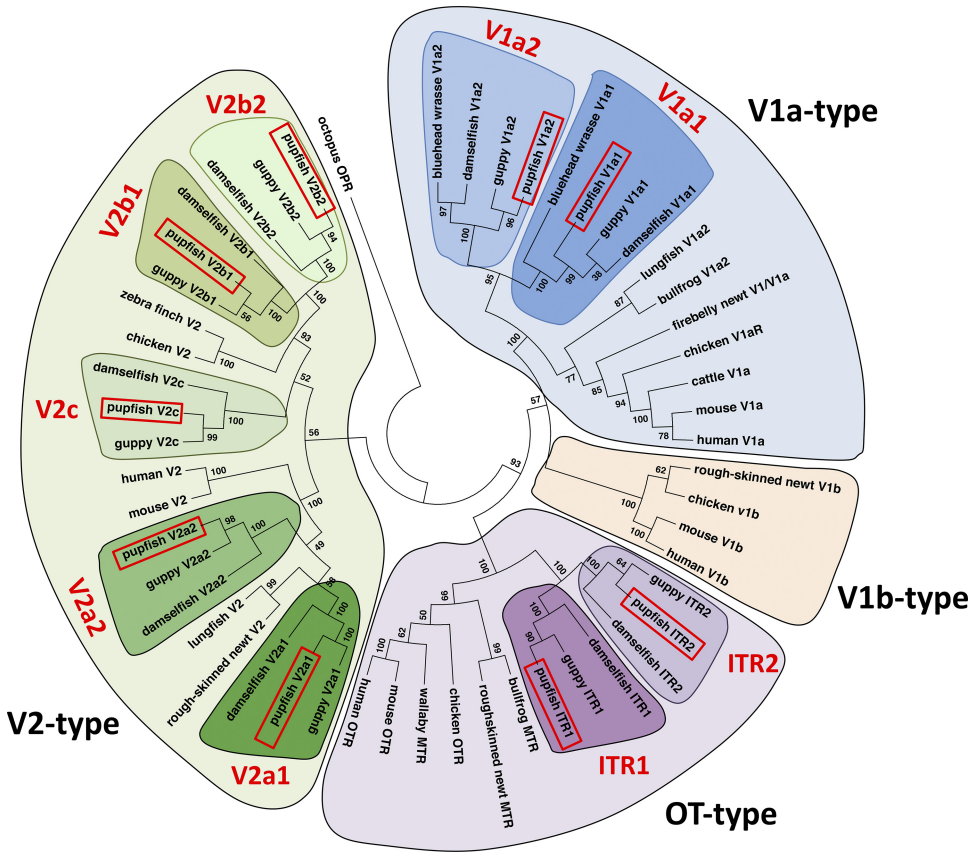

Fig. 1. Phylogenetic relationships among nonapeptide receptors in Amargosa pupfish (*Cyprinodon nevadensis*) and other selected vertebrates. Tree topology inferred using the neighbor-joining method from a deduced amino acid sequence alignment and bootstrapped with 1,000 replicates. Nonapeptide receptors are grouped into the V1a-, V1b-, and V2-type arginine vasopressin/arginine vasotocin (AVP/AVT) receptors, and receptors for oxytocin (OTR)/mesotocin (MTR)/isotocin (ITR). The nine putative nonapeptide receptors of Amargosa pupfish examined in the current study are indicated by red boxes. The octopus octopressin receptor (OPR; GenBank accession no. AB116233) was used to root the tree. Sequence accession ID numbers are as follows: Amargosa pupfish: V1a1 (GQ981412), V1a2 (GQ981413), V2a1 (GQ981414), V2a2 (JSUU01000639), V2b1 (KJ668598), V2b2 (JSUU01002888), V2c (JSUU01002888), ITR1 (GQ981415) and ITR2 (JSUU01008219); guppy, *Poecilia reticulata*: V1a1 (XM_008400322), V1a2 (XM_008411145), V2a1 (XM_008413598), V2a2 (XM_008414623), V2b1 (XM_008409900), V2b2 (XM_008410458), V2c (XM_008401187), ITR1 (XM_008408733) and ITR2 (XM_008409849); bicolor damselfish, *Stegastes partitus*: V1a1 (XM_008291654), V1a2 (XM_008284006), V2a1 (XM_008290597), V2a2 (XM_008290173), V2b1 (XM_008294366), V2b2 (XM_008279232), V2c (XM_008298062), ITR1 (NM_001294182) and ITR2 (NM_001294184); bluehead wrasse, *Thalassoma bifasciatum*: V1a1 (JQ639049) and V1a2 (JQ639050); lungfish, *Protopterus annectens*: V1a (AB377531) and V2 (AB377532); bullfrog, *Rana catesbeiana*: V1a (AY277924) and MTR (AY277925); firebelly newt, *Cynops pyrrhogaster*: V1/V1a (AB274037); rough-skinned newt, *Taricha granulosa*: V1b (EF567079), V2 (EF567078), and MTR (DQ186599); chicken, *Gallus gallus*: V1aR (NM_001110438 and EU124684), V1b (NM_001031498), V2 (NM_001031479), and MTR (NM_001031569); zebra finch, *Taeniopygia guttata*: V2 (XM_002195382); wallaby, *Macropus eugenii*: MTR (AY206419); cattle, *Bos taurus*: V1a (NM_00104990); mouse, *Mus musculus*: V1a (NM_016847), V2 (NM_019404) and OTR (NM_001081147); human, *Homo sapiens*: V1a (NM_000706), V1b (NM_000707), V2 (NM_000054) and OTR (NM_000916).

relative abundance 4 days after exposing pupfish to freshwater (0.3 ppt) and returned to control levels by 14 days.

Transcripts encoding the V2-type receptor *v2a1* in the gill also varied in relative abundance after salinity change (Fig. 3*C*) (treatment × time interaction: $F_{12,140} = 2.06$, $P = 0.024$), with *v2a1* transcripts being reduced in relative abundance 24 h and 4 days after transfer to freshwater. Salinity change did not alter gill mRNA levels for any of the other pupfish V2-type receptors (*v2a2*, *v2b1*, *v2b2*, or *v2c*). Similarly, salinity change did not alter gill mRNA levels for either pupfish IT receptor *itr1* or *itr2*.

Transcripts encoding the AVT/IT degradation enzyme *lnpep* were stable in relative abundance in the gill of pupfish maintained at 7.5 ppt (control) for 14 days but varied among pupfish transitioned to other salinities (Fig. 3*D*; treatment × time interaction: $F_{12,140} = 3.34$, $P = 0.0003$). At 4 days following salinity change, *lnpep* mRNAs were lower in relative abundance in the gill of fish exposed to freshwater (0.3 ppt) compared with fish in 35 or 55 ppt, indicating a decrease in gill *lnpep* mRNA levels following transfer of fish to hypoosmotic conditions and an increase following transfer to hyperosmotic conditions. These salinity-associated differences in *lnpep* mRNA levels, however, were no longer detected 14 days after salinity change.

Effects of AVT System Modulation on Osmolality and Gill Ion Transporter Expression

AVT system modulation via administration of the V1-type receptor antagonist Manning compound altered the extent of salinity-induced changes in plasma osmolality (salinity × injection treatment interaction: $F_{4,76} = 3.45$, $P = 0.0121$; Fig. 4). Mean osmolality values for pupfish from all injection treatments combined decreased from 366 ± 5.2 mosmol/kg

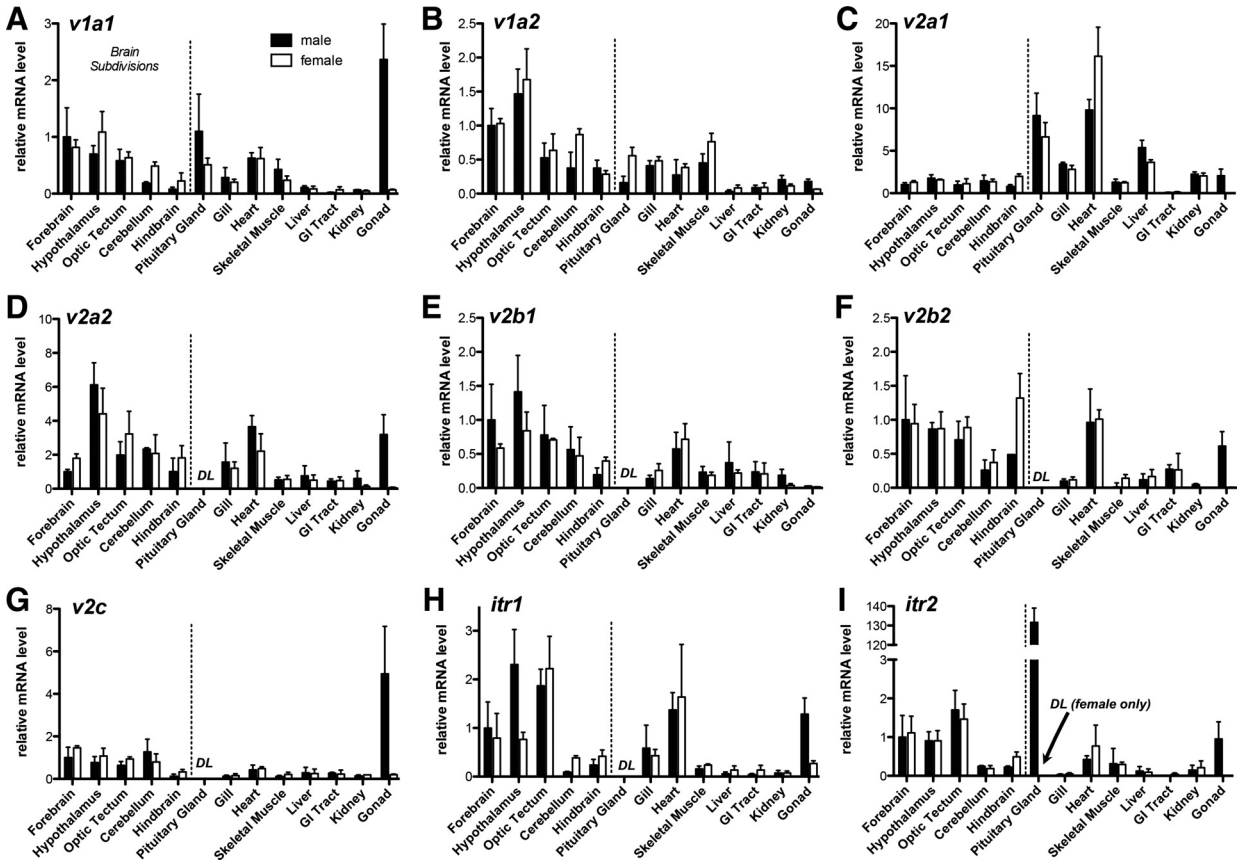

Fig. 2. Relative gene transcript abundance for the two arginine vasotocin (AVT) V1a-type receptors *v1a1* (*A*) and *v1a2* (*B*) and five V2-type receptors *v2a1, v2a2, v2b1, v2b2,* and *v2c* (*C–G*), as well as for the two isotocin (IT) receptors *itr1* (*H*) and itr2 (*I*), as determined by reverse transcription quantitative PCR. Data are shown as means ± SE. *DL* indicates that relative levels of that transcript were below detection limits in that tissue.

H_2O (mean ± SE) for pupfish maintained under the 7.5 ppt (control) salinity to 334 ± 5.7 mosmol/kg H_2O in fish transferred to 0.4 ppt, and increased to 473 ± 10.5 mosmol/kg H_2O in pupfish transferred to 35 ppt (one-factor ANOVA model using all injection treatments combined: $F_{2,82} = 83.42$, $P < 0.0001$). Modulation of the AVT system by administration of the V1-type receptor antagonist, however, increased plasma osmolality when pupfish were transferred to a higher salinity. Plasma osmolality was 477 ± 12.4 mosmol/kg H_2O in pupfish injected with the saline solution (control) and transferred to 35 ppt but increased further to 524 ± 29.1 mosmol/kg H_2O in pupfish injected with the V1-type receptor antagonist and then transferred to 35 ppt (Fig. 4). Modulation of the AVT system did not alter osmolality in pupfish exposed to either the freshwater (0.4 ppt) environment or maintained at the brackish 7.5 ppt control conditions.

Despite changes in plasma osmolality, gill NKA activity was not affected by either salinity ($F_{4,82} = 1.03$, $P = 0.3630$) or injection treatment ($F_{4,82} = 0.58$, $P = 0.5599$) over the 24-h period of exposure (Fig. 5*A*), suggesting that AVT system-mediated impacts on osmolality over this period did not involve changes in gill NKA. Supporting this idea, gene transcripts encoding the α_{1a}-subunit of NKA (*nka*α_{1a}) were also unaffected by AVT system modulation under all salinity conditions (Fig. 5*B*) despite mRNA levels for *nka*α_{1a} being elevated in the gill of pupfish transferred to 35 ppt ($F_{2,88} = 24.89$, $P < 0.0001$).

Gene transcript abundances for several ion channels or cotransporters were observed to increase in the gill of pupfish experiencing a change in environmental salinity, and some of these transcriptional changes were impacted by AVT system modulation. Transcript levels encoding the cystic fibrosis transmembrane conductance regulator (Cftr) Cl^- channel (*cftr*) varied with both salinity and AVT system modulation (salinity × injection treatment interaction: $F_{4,81} = 6.894$, $P < 0.0001$; Fig. 6*A*), with the V1-type receptor antagonism reducing gill *cftr* induction following transfer of pupfish to 35 ppt. Gill mRNAs for the teleost electrogenic Na^+-HCO_3^- cotransporter 1 (NBCe1) protein *nbce1.1* (Fig. 6*B*) and Na^+/H^+ exchanger 3 (*nhe3*) (Fig. 6*C*) also increased following transfer of fish to 35 ppt. That salinity-induced increase in *nbce1.1* mRNA levels, however, was reduced by administration of the V1-type receptor antagonist Manning compound (salinity × injection treatment interaction: $F_{4,81} = 3.49$, $P = 0.0111$; Fig. 6*B*). V1-type receptor antagonism likewise reduced the upregulation of gill *nhe3* expression after transfer to seawater (Fig. 6*C*; salinity × injection treatment interaction: $F_{4,81} = 4.54$, $P = 0.0023$). Transcript levels for the gill isoform of the Na^+/Cl^- cotransporter Ncc2 (*ncc2*), which mediates Na^+ and Cl^- uptake across the gill in freshwater, were also affected by modulation of AVT signaling (Fig. 6*D*). Transcripts for *ncc2* were elevated under control (7.5 ppt) conditions in pupfish that were treated with the V1-type receptor antagonist. Fish trans-

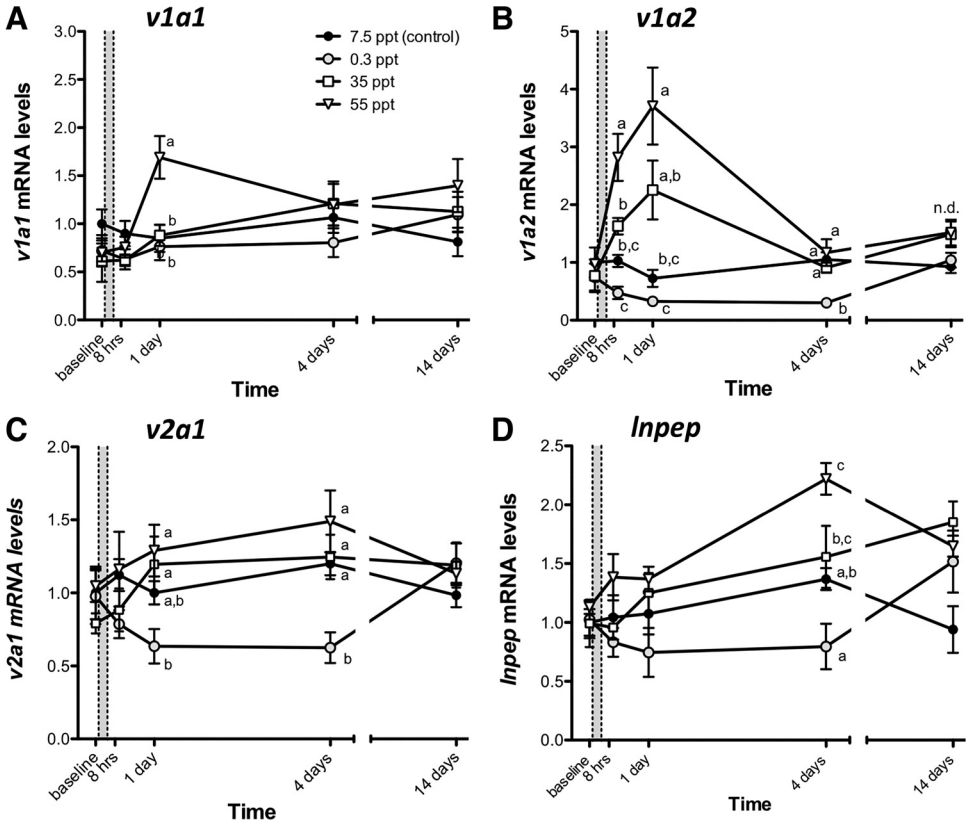

Fig. 3. Gill mRNA levels for teleost arginine vasotocin (AVT) V1a-type receptors *v1a1* (*A*) and *v1a2* (*B*), the V2-type receptor *v2a1* (*C*), and nonapeptide degradation enzyme leucyl-cystinyl aminopeptidase (*lnpep*; *D*) in pupfish following transfer from 7.5 ppt to salinities of 35 ppt, 55 ppt, or 0.3 ppt freshwater. Control fish were maintained at 7.5 ppt. Vertical gray area enclosed by dotted lines indicates period of salinity transition (4 h). Data are scaled relative to the control at the baseline (0 h) time point and are plotted as means ± SE; sample sizes are $n = 8$ fish per sampling time and treatment. Letters indicate significant pairwise differences between treatments within a given sampling time (Tukey honestly significant difference tests). Note *y*-axis scales differ among transcripts.

ferred to 0.4 ppt exhibited increased gill mRNA levels for *ncc2*; however, exogenous AVT prevented this freshwater-induced increase (Fig. 6*D*).

Although not altered by experimental modulation of AVT signaling, several other gene transcripts in the gill were observed to change in relative abundance following salinity transfer. The relative abundance of gene transcripts encoding Na^+-K^+-$2Cl^-$ cotransporter-1 (*nkcc1*) increased in the gill after transfer to 35 ppt (salinity effect: $F_{4,81} = 40.17$, $P <$ 0.0001; Fig. 7*A*). However, *nkcc1* mRNA levels were not affected by either AVT or Manning compound. Transcripts for the teleost NBCe1 isoform *nbce1.2* were increased in the gill of pupfish after transfer to 35 ppt ($F_{2,80} = 18.20$, $P < 0.0001$) but again were not affected by modulation of the AVT system (Fig. 7*B*). NHE isoform-2 (*nhe2*) mRNA levels were unaffected by either salinity or AVT system modulation (Fig. 7*C*). Transcript abundance for the aquaporin 1 channel (*aqp1*) was also not affected by either salinity or AVT modulation (Fig. 7*D*), but mRNAs encoding aquaporin 3 (*aqp3*) increased prominently in the gill of fish transferred from 7.5 to 0.4 ppt and to a lesser, but still statistically significant, extent in fish transferred to 35 ppt (Fig. 7*E*; salinity effect: $F_{2,81} = 27.04$, $P < 0.0001$). These salinity-induced changes in gill *aqp3* mRNA levels, however, were unaltered by administration of either AVT or the V1-type receptor antagonist Manning compound.

We also observed that the relative abundance of mRNAs encoding *lnpep* in the gill was again altered by salinity conditions (Fig. 8; salinity effect: $F_{2,81} = 4.27$, $P = 0.0173$), with pupfish transferred to 0.4 ppt exhibiting lower gill *lnpep* transcript levels than fish transferred to 35 ppt. Neither AVT nor the V1-type receptor antagonist affected these salinity-dependent changes in gill *lnpep* mRNA levels.

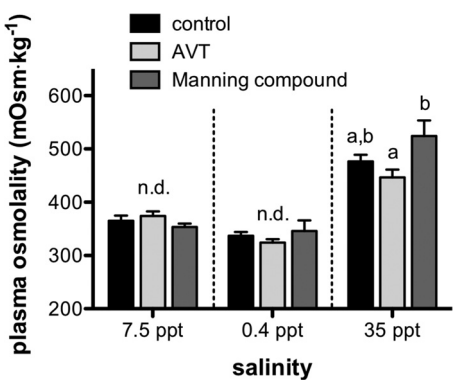

Fig. 4. Plasma osmolality (mosmol/kg H_2O) in pupfish acclimated to 7.5 ppt (control) and then injected with saline control, arginine vasotocin (AVT), or V1-type receptor antagonist Manning compound before transfer to 0.4 ppt (freshwater), 35 ppt (seawater), or maintenance at 7.5 ppt (salinity control). Osmolality increased in pupfish transferred to 35 ppt, and that increase was greater in fish treated with Manning compound than in AVT-treated fish. Data are shown as means ± SE; sample sizes are $n = 5$–10 per salinity and treatment. Letters indicate significant pairwise differences within a salinity treatment (Tukey honestly significant difference tests); n.d., no differences among treatments.

DISCUSSION

Evidence that AVT regulates osmotic balance in teleost fishes has been available for decades (6, 19), yet the mechanisms by which AVT exerts effects on osmoregulation by the gill have not been established. Recently, it was recognized that teleost fishes have evolved several nonapeptide receptors, including two V1a-type receptors, five V2-type receptors, and

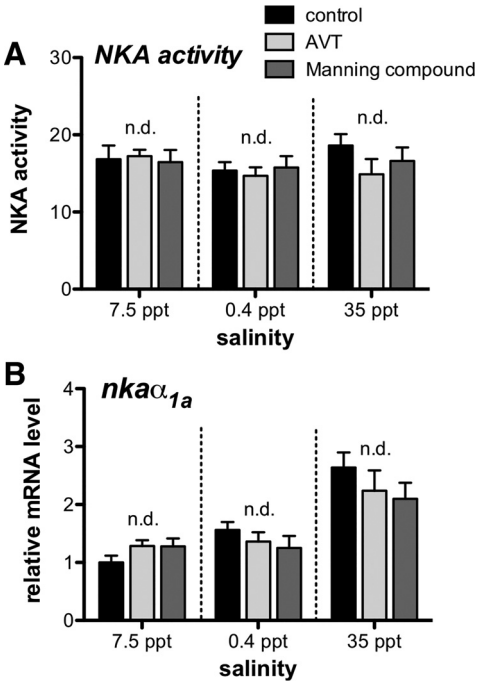

Fig. 5. Gill Na^+-K^+-ATPase (NKA) activity (*A*) and relative gene transcript abundance for the NKA α_{1a}-subunit (*nka*α_{1a}; *B*) measured 24 h after pupfish acclimated to 7.5 ppt were injected with saline control, arginine vasotocin (AVT), or V1-type receptor antagonist Manning compound and then transferred to 0.4 ppt (freshwater) or 35 ppt (seawater), or maintained at 7.5 ppt (salinity control). NKA activity was unaffected by salinity or hormone modulation. Transcript abundance for *nka*α_{1a} increased in fish moved to 35 ppt but was unaffected by AVT or Manning compound. Data are shown as means ± SE; $n = 10–11$ per salinity and treatment. Letters indicate significant pairwise differences within a salinity treatment (Tukey honestly significant difference tests); n.d., no differences among treatments.

two isotocin (IT) receptors (39, 44, 45, 74, 105), and which of these teleost receptors mediate the effects of AVT on the gill has been unclear. Here, we have begun to examine the role of these distinct teleost nonapeptide receptors in osmoregulation by the gill in the euryhaline Amargosa pupfish.

Transcriptional Responses Point to a Role for the V1a2 Receptor in Gill Osmoregulation

First, we examined transcriptional responses for all nine teleost nonapeptide receptors in the gill of pupfish following a rapid shift in environmental salinity. We found that the teleost *v1a1* and *v1a2* V1a-type receptors and the *v2a1* V2-type receptor exhibited changes in gill mRNA levels in patterns suggestive of a role for these receptors in salinity acclimation. Transcripts encoding the pupfish *v1a2* V1a-type receptor showed the clearest pattern of expressional regulation during salinity acclimation, with a two- to fourfold upregulation in the pupfish gill 24 h after transfer from brackish salinity conditions (7.5 ppt) to hypersaline environments of 35 or 55 ppt, respectively (Fig. 3*B*). This hypersalinity-induced elevation in gill *v1a2* mRNAs appeared dependent on the extent of environmental salinity increase and displayed a temporal pattern indicative of rapid (within 8 h) transcriptional upregulation followed by return to control levels within 4 days after salinity change. Pupfish exposed to freshwater (0.3 ppt) also showed evidence of decreased *v1a2* mRNA levels, although this reduction in *v1a2* mRNAs was not statistically significant until 4 days after the salinity change. These factors taken together, the pattern of relative gene transcript abundance variation observed in the pupfish *v1a2* gene supports the idea that changes in expression of the teleost V1a-type receptor V1a2 may contribute to the effects of AVT on the teleost gill during acclimation to both hyper- and hypoosmotic conditions.

The V1a2 V1a-type receptor of pupfish is homologous to the first AVT receptor that was isolated and sequenced from other teleost fishes (4, 14, 51, 100) and is highly expressed in several tissues, including the brain, heart, skeletal muscle, gonads, and gills (44, 45, 60). Our findings that *v1a2* receptor mRNAs are elevated in the gill epithelium following transfer of pupfish to higher salinities is consistent with the pattern observed in a prior, more limited study in pupfish, where gill *v1a2* mRNA levels also increased approximately twofold in pupfish transferred from 2.1-ppt to either 17- or 34-ppt environments (45). A hyperosmotic induction of gill *v1a2* transcription was also recently observed in the gilthead sea bream, *Sparus aurata*; transcripts encoding *v1a2* increased in abundance in sea bream 7 days after transfer of fish from 40 to 55 ppt and remained elevated even 14 days after the salinity change (60). Interestingly, increased gill *v1a2* mRNAs were also recently observed following transfer of freshwater-acclimated marbled eel, *Anguilla marmorata*, to brackfish (10 ppt) water but not to a higher salinity of 25 ppt (12).

Despite teleost fishes having evolved a second V1a-type receptor, our findings indicate the *v1a2* receptor as being the mostly likely V1a-type receptor for mediating the effects of AVT on the gill during salinity acclimation, based on the observed pattern and timing of transcript abundance changes in *the v1a2* when pupfish are exposed to a change in salinity. Even so, we also observed a less pronounced, but statistically significant, change in transcript abundance for the pupfish *v1a1* receptor. The teleost V1a-type receptor V1a1 is encoded by a gene paralog of the V1a2 receptor (39, 44, 45, 74, 105) that arose from duplication of a single V1a-type receptor gene (39). In the present study, gill *v1a1* mRNAs were ~70% elevated 24 h after transitioning pupfish from 7.5 ppt to a hypersaline, 55 ppt condition (Fig. 3*A*). However, no change in *v1a1* mRNA level was detected in pupfish transitioned to 35 ppt. To our knowledge, only one other study to date has examined gill *v1a1* transcription responses to salinity change. In that work, gill *v1a1* mRNAs declined ~40% in relative abundance 5 h after transfer from 2.1 to 17 ppt, returned to control levels by 20 h, and were not altered at all in the gill of fish transferred to a higher salinity (34 ppt) treatment (45). The present study also found little response of gill *v1a1* mRNA levels at normal seawater (35 ppt), but we did see increases at 55 ppt, but these changes were not nearly as great as the fourfold increase observed in gill *v1a2* mRNA levels (Fig. 3*B*). On the basis of patterns of *v1a1* mRNA variation in the hypothalamus, Lema et al. (43) hypothesized that the V1a1 receptor might function in a context related to general cellular or physiological responses to stressors. Additional studies are needed, however, to determine whether that hypothesis is valid. Transcripts for *v1a1* have been observed at greatest relative levels in the brain and testis of fishes (e.g., 33, 44, 45, 76). Given that our data here suggest that *v1a1* mRNA levels in the gill are less responsive to salinity change than *v1a2* mRNAs, the primary

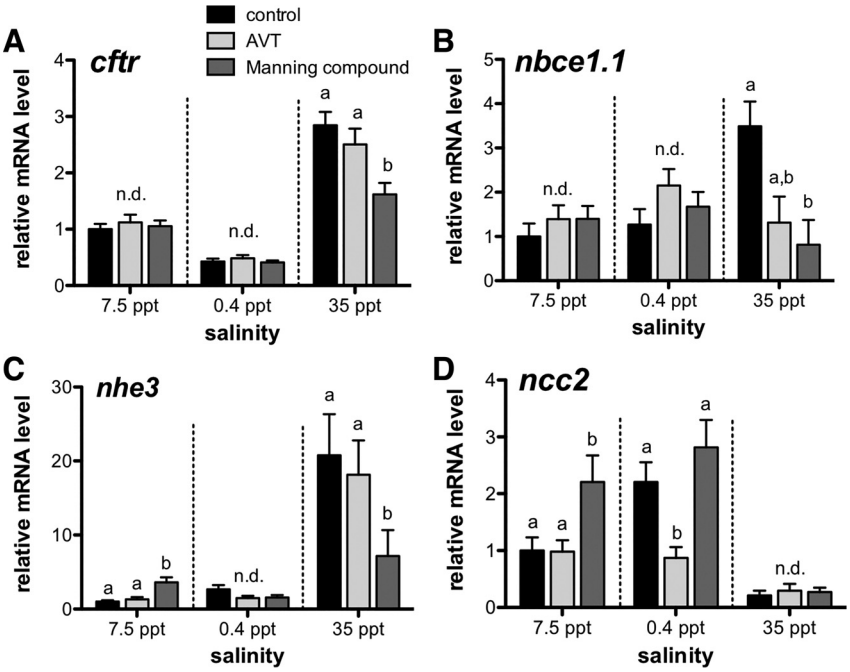

Fig. 6. Treatment with arginine vasotocin (AVT) or V1-type receptor antagonist Manning compound before transfer from 7.5 ppt (salinity control) to 0.4 ppt (freshwater) or 35 ppt (seawater) altered relative gene transcript abundance in the gill for cystic fibrosis transmembrane conductance regulator (*cftr*; *A*), Na^+-HCO_3^- cotransporter 1 isoform *nbce1.1*; *B*), Na^+/H^+ exchanger isoform 3 (*nhe3*; *C*), and Na^+-Cl^- cotransporter-2 (*ncc2*; *D*). Data are shown as means ± SE; n = 10–11 fish per treatment. Letters indicate significant pairwise differences within a salinity treatment (Tukey honestly significant difference tests); n.d., no differences among treatments.

function(s) of the teleost V1a1 V1a-type receptor paralog does not appear related to osmoregulation.

Alternatively, some of the action of AVT on V1a- or V2-type receptors in the gill may relate to the modulation of energetic pathways during osmoregulation. Previous work has indicated that energetic demands of osmoregulatory tissues can rise during the early phases of increases or decreases in external salinity (e.g., Ref. 92). Martos-Sitcha et al. (57) recently demonstrated that genes associated with energy metabolism (e.g., glycolysis, gluconeogenesis) were strongly upregulated in the gill of sea bream following transfer to both hypo- and hyperosmotic conditions, supporting the idea that osmoregulation in both low- and high-salinity environments is energetically demanding, at least during the early "adaptive" phase. Experimental studies using exogenous AVT provide evidence for AVT modulation of metabolic status. AVT treatment of sea bream increased plasma glucose following transfer of fish from seawater (36 ppt) to either lower-salinity (6 ppt) or hypersaline (55 ppt) conditions but not when fish remained in 36 ppt seawater (81). Intraperitoneal injection of AVT has also been shown to decrease glycogen content in the brain of rainbow trout in a dose-dependent manner (83) and to increase glucose and cortisol levels in the absence of salinity change (17). Such findings suggest that AVT may enhance glycolytic pathways in some tissues either directly, or indirectly via modulation of other hormones such as ACTH and/or cortisol (40, 82). Future studies using AVT receptor-specific agonists (i.e., felypressin, desmopressin) or antagonists [i.e., relcovaptan (SR-49059); tolvaptan] should be conducted to help distinguish which teleost nonapeptide receptor(s) mediates the action of AVT on cellular metabolism in the gill and other tissues during osmoregulation and whether metabolic actions of AVT occur independently of osmoregulatory demands.

Interestingly, we did not observe any changes in gill *itr1* or *itr2* mRNA levels following the transfer of pupfish to higher or lower salinities despite evidence that relative mRNA levels for *itr1* (termed *itr* in that study) declined in the gill of Amargosa pupfish 20 h after the transfer of wild juvenile pupfish from 2.1 to 34 ppt (45). Similar to our results here, Lema (45) observed *itr1* mRNAs to be expressed in the gill at moderate to low abundance, while our data here also indicate that *itr2* mRNAs are only at very low levels in the gill. The functional significance of that gill Itr1 expression remains unclear, and the differing results between our findings here and those of Lema (45) may related to differences in the age of pupfish used or influences from other stressors. Lema (45) collected wild juvenile pupfish from the Amargosa River and immediately transferred them from the river (2.1 ppt) directly to 17 or 34 ppt without any acclimation period after collection. It is very likely that pupfish in that study would have elevated glucocorticoid and epinephrine concentrations, which may have modulated gill *itr1* mRNA levels independently or in combination with other salinity-mediated physiological responses. Clearly, exploration of the effects of cortisol on *itr1* expression in the gill and other tissues to test for stress-associated modulation of IT signaling pathways is warranted in future studies.

Evidence That AVT Acts on a V1-Type Receptor to Modulate Gill Cl^- Transport

Given the patterns of gill *v1a2* mRNA abundance variation observed in pupfish during salinity acclimation, we further tested whether a V1-type receptor might mediate the effects of AVT on gill osmoregulation by administering a mammalian V1-type receptor antagonist (Manning compound) to pupfish before transfer from 7.5 ppt to 0.4 or 35 ppt. We found that intraperitoneal injection of this V1-type receptor antagonist resulted in a larger increase in plasma osmolality following transfer of pupfish to 35 ppt. Sangiao-Alvarellos et al. (82) observed that AVT modulated gill NKA activity in seawater-adapted sea bream, although the effects were dependent on salinity conditions and the duration of the AVT treatment.

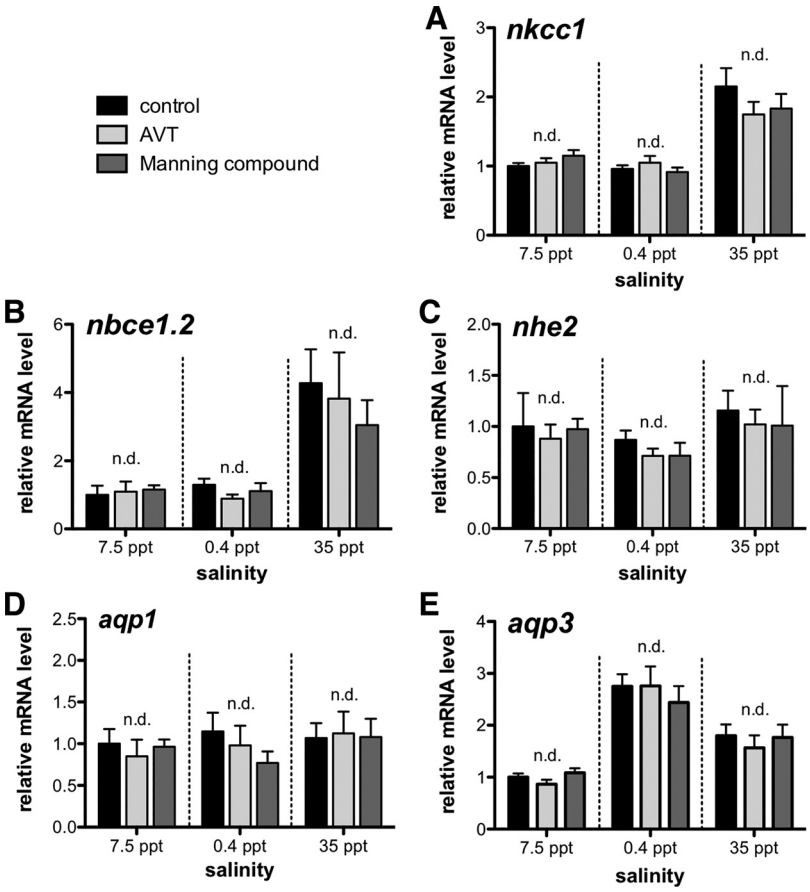

Fig. 7. Administration of arginine vasotocin (AVT) or V1-type receptor antagonist Manning compound had no effect on relative gene transcript abundance in the gill for Na^+-K^+-$2Cl^-$ cotransporter 1 (*nkcc1*; *A*), Na^+-HCO_3^- cotransporter 1 isoform *nhce1.2 (B)*, Na^+/H^+ exchanger isoform 2 (*nhe2*; *C*), or either aquaporin-1 (*aqp1*; *D*) or aquaporin-3 (*aqp3*; *E*). Data are shown as means ± SE; sample sizes are $n = 10–11$ per salinity and treatment; n.d., no differences among treatments.

Seawater-acclimated (36 ppt) sea bream injected with AVT at the same dose used here with pupfish (1 μg AVT/g body mass) showed no changes in gill NKA activity even when transferred to higher or lower salinities, whereas fish treated chronically with AVT implants showed elevated gill NKA activity after 3 days in the absence of any salinity change (82). Pupfish treated with either AVT or the V1-type antagonist did not show changes in either NKA activity or NKA α_{1a}-subunit mRNA levels after 24 h (Fig. 5), an observation consistent with the findings in AVT-injected sea bream (82). AVT system modulation was, however, observed to alter the pattern of gene transcript regulation in pupfish for two Cl^- transport proteins: Na^+-Cl^- cotransporter (Ncc2) and the Cftr Cl^- channel.

Gill Ncc2 expression has been proposed to be involved in Cl^- and Na^+ uptake during hypoosmotic stresses (26, 98), and increases in gill *ncc2* gene expression have been observed previously in tilapia, *Oreochromis mossambicus*, during acclimation from higher- to lower-salinity conditions (29, 50, 69, 70). Prior investigations with Amargosa pupfish showed that fish transferred from 7.5 ppt to higher salinities (35 or 55 ppt) showed decreased gill *ncc2* mRNA levels within 8 h and that gill *ncc2* mRNA levels increased in pupfish during acclimation to freshwater (0.3 ppt) (42). Our data here are consistent with those previously documented patterns of salinity-induced gill *ncc2* mRNA regulation, with saline-injected (control) pupfish exhibiting lower gill *ncc2* mRNAs 24 h after transfer to 35 ppt (Fig. 6*D*). Our results, however, also provide evidence for AVT modulation of those salinity-dependent changes in gill *ncc2* expression, as the V1-type receptor antagonist increased gill *ncc2* mRNA levels under control (7.5 ppt) conditions, whereas AVT reduced gill *ncc2* in fish acclimating to freshwater. Although it remains unclear how these *ncc2* mRNA abundance changes might relate to Ncc2 protein expression,

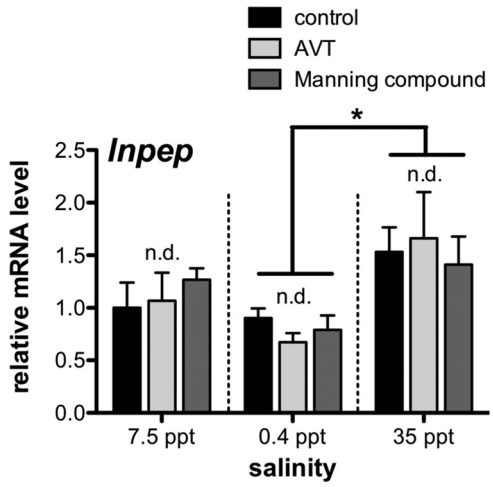

Fig. 8. Gill mRNA levels for leucyl-cystinyl aminopeptidase (*lnpep*) in pupfish acclimated to 7.5 ppt and then injected with arginine vasotocin (AVT) or V1 receptor antagonist Manning compound before transfer to 0.4 ppt or 35 ppt. Although neither AVT nor Manning compound affected *lnpep* mRNA responses, fish transferred to 35 ppt showed elevated gill *lnpep* mRNA levels compared with those transferred to 0.4 ppt (*); n.d., no differences among injection groups within that salinity treatment. Data are shown as means ± SE; $n = 10–11$ per salinity and treatment; n.d. indicates no pairwise differences among treatments.

the pattern of transcriptional regulation of *ncc2* suggests that AVT's actions on the gill might be, in part, to reduce Cl^- and Na^+ uptake in low-salinity environments.

The Cftr Cl^- channel is expressed apically in gill ionocytes where it facilitates Cl^- secretion to the external environment (31, 55, 56, 66). That apical secretion of Cl^- by Cftr occurs in conjunction with Cl^- movement into the ionocyte from the blood by Nkcc1, which is positioned basolaterally in ionocytes. Gill *cftr* mRNA levels have previously been shown to increase four- to fivefold in pupfish within 8 h of transfer from 7.5 ppt to higher salinities (35 ppt or 55 ppt) (42), which is consistent with our observation here of an increase in *cftr* mRNA levels in pupfish transferred from 7.5 ppt to 35 ppt. That pattern of hyperosmotic *cftr* upregulation is also consistent with results seen with a related cyprinodontoid fish, the mummichog (*Fundulus heteroclitus*), as well as several other fishes, following the transfer of fish from lower to higher salinities (e.g., 10, 26, 30, 85, 86, 87). In the present study, however, we also observed that treatment with the V1-type receptor antagonist Manning compound inhibited the increase in gill *cftr* mRNA levels as pupfish acclimate to hyper-saline conditions. Based on that finding, it is likely that a V1-type receptor normally contributes – directly or indirectly – to increased gill Cftr expression after seawater exposure; in the presence of a V1-type receptor antagonist this increase in Cftr expression is inhibited, resulting in a reduced capacity for ion secretion and the higher plasma osmolality levels observed in the present study.

Based on the patterns of AVT system modulation of gill *ncc2* and *cftr* mRNA levels observed here, our data support the hypothesized role of AVT as a modulator of gill Cl^- transport. Specifically, by reducing Ncc2 expression, AVT may act to reduce gill Cl^- uptake from the environment, while also acting to increase Cl^- secretion by supporting the induction of gill Cftr expression when fish experience hyperosmotic conditions. This proposed mechanism of action for AVT is generally consistent with a model proposed recently based on studies in sea bream (52). Unlike those studies in sea bream, however, we have not detected any changes in NKA activity or NKA subunit mRNA levels linked to AVT signaling. Instead, our data here in pupfish indicate that AVT may act on the gill to decrease gill Ncc2 expression, thereby pointing to a potentially new mechanism wherein AVT regulates Cl^- transport in low salinity environments.

In addition, we also observed that V1-type receptor inhibition reduced salinity-associated induction of gill *nbce1.1* and *nhe3* mRNA levels in pupfish transferred from 7.5 ppt to 35 ppt (Fig. 6, *B* and *C*). Recent work in this same pupfish species identified two distinct NBCe isoforms, *nbce1.1* and *nbce1.2*, and provided evidence that these isoforms have dissimilar transcriptional response patterns in the gill during salinity acclimation (42). In that previous work, transcripts encoding *nbce1.1* were observed to increase six- to eightfold in the gill within 24 h of transferring pupfish from 7.5 ppt to hypersaline environments (35 or 55 ppt), and *nbce1.1* mRNA levels remained elevated as much as 22-fold even 14 days after salinity transfer. Transcripts for *nbce1.2*, on the other hand, increased in the gill of pupfish transferred from 7.5 ppt to 0.3 ppt (42). Although little is known about the functional differences between the two isoforms, basolateral NBCe1 has been implicated in acid-base regulation as well as basolateral Na^+ movement from the cytoplasm to serosal side of ionocytes in fishes in low-salinity environments (75). The inhibition of *nbce1.1* transcriptional induction by Manning compound therefore suggests that AVT signaling may influence acid-base regulation by the gill. Further supporting this idea, AVT appears to alter gill Nhe3 expression, either directly or indirectly, based on our observations that treating fish with Manning compound both impairs the increase in gill *nhe3* mRNAs caused by transition to a hyperosmotic environment and induces a change in *nhe3* mRNAs in pupfish maintained under control (7.5 ppt) conditions. In teleost fish, gill Na^+/H^+ exchangers, including Nhe2 and Nhe3, appear to facilitate Na^+ influx in freshwater (15, 86) but may also be involved in acid-base regulation, as gill Nhe3 protein levels have been observed to increase in seawater-adapted mummichog exposed to hypercapnic conditions (15).

More broadly, our findings herein provide additional support for the general model for salinity-induced AVT effects as outlined by Mancera et al. (52). Pupfish exposed to higher-salinity conditions decrease hypothalamic AVT expression, as indicated by reductions in the relative abundance of AVT mRNAs in the hypothalamus (45) and by reductions in the number of AVT-ir neurons in the magnocellular region of the hypothalamic preoptic area (47, 48). Those changes in hypothalamic AVT mRNA levels and magnocellular AVT-ir neuron density in pupfish appear consistent with what has been observed in other fishes following transfer to high-salinity conditions (23, 27, 28, 52, 73). Together, reductions in hypothalamic AVT expression and increases in gill V1a2 receptor expression may be one part of a coordinated response of increased neurohypophysis AVT secretion and AVT action on the gills to enhance Cl^- secretion as fish acclimate to high-salinity environments.

In the present study, we also observed salinity-dependent changes in gill *lnpep* mRNA levels (Fig. 7), which may indicate changes in LNPEP enzymatic activity to facilitate the degradation of AVT in at least some cells within the gill epithelium. The LNPEP enzyme belongs to the M1 subfamily of AVP/OT-degrading aminopeptidases that was first identified in the mammalian placenta (68, 93, 94); only very recently were homologous forms of LNPEP identified in other vertebrates (16). Transcripts encoding *lnpep* have been previously detected in the gill of pupfish (16), but it remains unclear whether these transcripts are translated into functional enzymes or to which cell type(s) LNPEP localizes within the gill epithelium. Nonetheless, our finding here that *lnpep* mRNAs increase in relative abundance in the gill of pupfish acclimating to elevated salinities and decrease in fish acclimating to lower salinities, points to a possible role of changes in LNPEP expression as an additional component in modulating the availability of AVT, IT, or other small peptides in the gill during salinity acclimation.

It is important to note, however, that the osmolality consequences of in vivo V1-type receptor antagonism observed here could have been mediated not only by action on the V1-type receptors in the gill but also in other tissues involved in osmotic balance such as the intestines, skin, or kidney. For instance, V1a2-type receptor mRNAs are present in the intestines of fish (45, 59, 61), and it is likely that the intraperitoneal administration of Manning compound concurrently altered AVT's influences on water absorption or ion secretion in that tissue along with the gill, if those effects were mediated via V1-type receptor activation.

Furthermore, we also observed a decline in V2-type *v2a1* mRNA levels in the pupfish gill 24 h after transition to a freshwater environment. Transcript abundance for this *v2a1* receptor remained lower at 4 days but returned to baseline levels by 14 days following salinity transition. On the basis of studies of sea bream, Mancera et al. (52) proposed a role for a V2-type receptor in mediating salinity-associated changes in gill Cftr expression. In sea bream, gene transcripts for a V2a-type receptor have been observed to decline in relative abundance 3 days following transfer of fish from 40 ppt to 5 ppt salinity and increase in fish transferred from 40 ppt to 55 ppt (60). Although we did not conduct any pharmacological manipulations of V2-type receptor signaling in the current study, Martos-Sitcha et al. (58) observed that administration of the V2-type receptor antagonist Tolvaptan inhibited AVT-induced gill Cl^- secretion in the mummichog. It is therefore possible that the decline in *v2a1* mRNA levels that we observed in pupfish exposed to 0.3 ppt might indicate a reduction in gill V2a1 receptor expression so as to reduce the sensitivity of the gill to AVT-mediated Cl^- secretion as fish acclimate to a hypoosmotic environment. However, our data showing modulation of the salinity-induced increase in gill *cftr* mRNAs implies that Cftr expression is regulated, at least in part, via a V1-type receptor pathway instead of, or in addition to, a V2-type receptor pathway.

Despite previous studies in teleost fishes providing evidence that IT can influence osmotic balance (52), we did not observe any salinity-induced changes in mRNA levels of either pupfish ITR gene. In adult mummichog, exogenous IT was found to stimulate gill Cl^- secretion; however, IT had no effect on gill Cl^- transport in sea bream, indicating that the effects of IT on gill ionoregulation may vary among fish taxa (58). Gene transcript abundance for an ITR in the gill has also been shown in freshwater-acclimated marbled eel to increase following transfer to brackish water (10 ppt) but to decline after transfer to 25 ppt (12), implying a possible role for an ITR in gill salinity acclimation. Alternatively, the effects of IT on fish osmoregulation may occur largely via action on tissues other than the gills. In zebrafish (*Danio rerio*), for instance, overexpression of the IT gene enhances ionocyte proliferation in zebrafish skin (13). In sea bream, IT has been suggested to regulate aquaporin 1 function and water absorption within the intestine (59, 61), where ITR mRNA levels have also been shown to change during salinity acclimation in some species (12).

Based on recent observations of AVT and IT signaling modulation of gill Cl^- secretion in mummichog and sea bream (58), it is also possible, and perhaps even likely, that that action of AVT and IT on the gill may differ across taxa (54, 84) as well as across ecological contexts (e.g., cycling salinity environments, prior salinity experience). Evidence for species variation in the effects of AVT support the possibility of diversity in nonapeptide functions in osmoregulation across fishes (37, 58). The regulation of gill permeability and ion transport by AVT/IT may therefore vary among taxa, and attention to the possibility of such evolutionary differences including potential species differences in gill nonapeptide receptor expression and function should drive future investigations in a variety of euryhaline and stenohaline fishes.

Perspectives and Significance

For the first time, gene expression responses for all nine teleost nonapeptide receptors were examined in the gill epithelium during salinity acclimation. The pattern of transcript abundance changes for the pupfish V1a2 receptor after transfer of fish to higher or lower salinities pointed to a role for this V1a-type receptor in osmoregulation. V1-type receptor antagonism was observed to alter gill *cftr* and *ncc2* transcriptional responses during acclimation of fish to altered salinities. These findings provide evidence that the teleost V1a2 receptor may regulate gill Cl^- transport by inhibiting Cl^- uptake and facilitating Cl^- secretion during seawater acclimation.

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DISCLAIMERS

Fish for these experiments were collected under permission of the California Department of Fish and Wildlife (Scientific Collecting Permit no. SC-4793). Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the US Government.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.C.L., E.H.W., and M.E.C. conceived and designed research; S.C.L., E.H.W., M.E.C., P.G.C., J.N.E., and S.D.M. performed experiments; S.C.L., E.H.W., M.E.C., P.G.C., and J.N.E. analyzed data; S.C.L., E.H.W., M.E.C., and S.D.M. interpreted results of experiments; S.C.L. prepared figures; S.C.L. drafted manuscript; S.C.L. and S.D.M. edited and revised manuscript; S.C.L., E.H.W., M.E.C., P.G.C., J.N.E., and S.D.M. approved final version of manuscript.

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