



Altered expression of Na⁺/K⁺-ATPase and other osmoregulatory genes in the gills of euryhaline animals in response to salinity transfer: A meta-analysis of 59 quantitative PCR studies over 10 years



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ABSTRACT

Recent advances in molecular techniques have allowed gene expression in euryhaline animals to be quantified during salinity transfers. As these investigations transition from studying single genes to utilizing genomics-based methodologies, it is an appropriate time to summarize single gene studies. Therefore, a meta-analysis was performed on 59 published studies that used quantitative polymerase chain reaction (qPCR) to examine expression of osmoregulatory genes (the Na⁺/K⁺-ATPase, NKA; the Na⁺/K⁺/2Cl⁻ cotransporter, NKCC; carbonic anhydrase, CA; the cystic fibrosis transmembrane regulator, CFTR; and the H⁺-ATPase, HAT) in response to salinity transfer. Based on 887 calculated effect sizes, NKA, NKCC, CA, and HAT are up-regulated after salinity transfer, while surprisingly, CFTR is unchanged. Meta-analysis also identified influential factors contributing to these changes. For example, expression was highest: 1) during transfers from higher to lower salinities comprising a physiological transition from osmoconformity to osmoregulation, 2) 1–3 days following transfer, 3) during dissimilar transfers, and 4) in crustaceans rather than teleosts. Methodological characteristics (e.g., types of controls) were not important. Experiments lacking in the current literature were also identified. Meta-analyses are powerful tools for quantitatively synthesizing a large body of literature, and this report serves as a template for their application in other areas of comparative physiology.

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1. Introduction

Advances in molecular techniques for non-model organisms and the popularity of the quantitative polymerase chain reaction (qPCR) have allowed comparative physiologists to study the expression of single proteins of interest in the gills of euryhaline animals during salinity transfer, with the first such studies being published about 10 years ago (Towle et al., 2001; Lucu and Towle, 2003; Richards et al., 2003). The expansion of a quantitative molecular approach allowed researchers to work with the genes encoding transport proteins, instead of the proteins themselves, which are often large, trans-membrane molecules that are difficult to study using more traditional biochemical methods. Importantly, these new molecular techniques allowed researchers to identify more transport proteins in the gills, characterize their functional properties, and study regulatory mechanisms responsible for the increases in transport protein activity seen in earlier studies

(e.g., transcriptional vs. post-transcriptional modification; for a recent comprehensive review see Henry et al., 2012).

Although qPCR continues to be a popular technique for investigating individual genes of interest (e.g., Gilmour et al., 2012; Loong et al., 2012; Wang et al., 2012), a new molecular revolution is currently underway, and physiologists are now beginning to use genomic-based approaches that simultaneously examine a significant portion of the transcriptome. This is due to the advent of microarray technology (e.g., Towle et al., 2011) and more recently, next generation sequencing (e.g., Trapnell et al., 2010; Lowe et al., 2011; Meyer et al., 2011). As sequencing costs decline, this field may continue to shift to a more genomics and less single-gene based approach. Therefore, it may be a good time to summarize the current literature on how salinity affects gene expression based on studies using qPCR. Meta-analysis is one way to quantitatively and statistically synthesize many such studies (Glass, 1976). Although not typically appearing in comparative physiology journals, meta-analysis is popular in the fields of ecology (Cardinale et al., 2006), medical genomics (Gieger et al., 2011), cell biology (Broadhead et al., 2006), and global climate change (Parmesan and Yohe, 2003). Meta-analyses have often been used to summarize many inconclusive medical studies (where large sample sizes are impossible) to give a more robust conclusion, but meta-analyses can also summarize the state of knowledge

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within a field, highlight future areas of research, and point out factors leading to heterogeneity in studies (i.e., why do similar studies get different results?). These later purposes seem more applicable to studies of salinity-induced gene expression, as it is not controversial whether long-studied genes and proteins are important in ion-transport, but under what scenarios deviations from the norm may be expected.

The Na^+/K^+ -ATPase (NKA) has been the most popular gene examined in osmoregulatory studies, in terms of number of studies published (e.g., 76% of the studies analyzed here). This is not surprising, as the NKA is a critical component in osmotic/ionic regulation (for reviews, see Towle et al., 1976; Shoemaker and Nagy, 1977; Evans, 2008; Henry et al., 2012). The NKA is responsible for establishing electrochemical gradients across the cell membrane in the gills of euryhaline animals and has been found in the gills of all such species examined. Other genes have also been implicated in osmoregulation and work in concert with the NKA to actively secrete or absorb ions across the gills. These include: 1) the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC), which transports ions into gill cells either from the blood or environment based on salinity (Riessenpatt et al., 1996; Towle and Weihrauch, 2001; Hwang and Lee, 2007), 2) the H^+ -ATPase (HAT), which pumps protons to influence HCO_3^- and Cl^- exchange and Na^+ uptake (Weihrauch et al., 2004; Tresguerres et al., 2006), 3) carbonic anhydrase (CA), which produces the H^+ and HCO_3^- needed to drive coordinated Na^+ and Cl^- exchange (Henry and Cameron, 1983; Hirata et al., 2003), and 4) the cystic fibrosis transmembrane regulator (CFTR), a Cl^- channel that has mainly been studied in euryhaline fish osmoregulation (Singer et al., 1998). Other genes such as the Na^+/H^+ exchanger (Edwards et al., 1999) and the $\text{Na}^+/\text{HCO}_3^-$ cotransporter (Hirata et al., 2003) have also been implicated in this process, although their expression patterns have not been analyzed as thoroughly.

Because studies examining expression of single genes during salinity transfer have been accumulating for the last ~10 years, they contain a large but manageable amount of data. To summarize these data, we performed a meta-analysis of 59 published studies investigating changes in expression of five genes in response to salinity transfer redundant in euryhaline animals. Studies of NKA provided the most robust data set, although studies investigating NKCC, CA, CFTR, and HAT were also examined. The objectives of this meta-analysis were to: 1) identify the causes of differences in gene expression between studies based on differing methodologies and experimental designs (i.e., heterogeneity), 2) identify experiments lacking in the literature in an effort to fill in research deficiencies and direct future research, and 3) provide a database that can be used by researchers to design future experiments and compare their results to what is already known. It is hoped that this meta-analysis will prompt similar studies in related areas such as acid–base regulation, ammonia excretion, metabolism, as well as for other genes of interest.

2. Materials and methods

2.1. Data acquisition

Literature searches were performed including all published studies through mid-2012 using PubMed and Web of Science databases to identify published papers that met the search criteria. These criteria were: 1) Study organisms had to be euryhaline metazoans (not plants or microorganisms), 2) Expression had to be measured via relative mRNA abundance (i.e., not protein-based or enzyme activity based) using qPCR (i.e., “semi-quantitative” gel-based methods were not included), 3) A mean for a control and treatment condition had to be measurable, and 4) Expression had to be measured in the gills, as they are the major site of osmoregulation and the most-studied osmoregulatory tissue in euryhaline animals (Evans et al., 2005; Henry et al., 2012). Although all journals were considered during initial literature searches, later searches focused on a number of specific journals that tended to publish studies

meeting the search criteria: *The Journal of Experimental Biology*, *Comparative Biochemistry and Physiology A, B, & D*, *The American Journal of Physiology*, *The Journal of General and Comparative Endocrinology*, and *The Journal of Experimental Zoology*. Search terms initially used were combinations of the following: salinity, transfer, gene expression, qPCR, mRNA, gene names and acronyms, and gill. After initial searches, reference-based searching was used.

2.2. Details of the meta-analysis

Effect sizes (values representing the magnitude of an experimental manipulation on a certain measurement) were calculated for each experiment in each paper by comparing relative gene expression values gleaned from figures and tables between a “control state” and a “treatment state”. The control state consisted, when possible, of expression values calculated for “sham” transfers of animals into the same salinity to which they were chronically acclimated. Treatment states consisted of experimental expression values for transfers to a different salinity taken at the same time points as in the sham transfers. When sham transfers were not performed (63% of the effect sizes), control values were calculated before transfers at a zero-time point (i.e., a pre-transfer expression value). These types of comparisons were often performed in the original studies and are not unique to this meta-analysis.

In a meta-analysis, effect sizes are compared using a common effect size metric. There are over 60 common effect size metrics to consider when conducting a meta-analysis (Huberty, 2002), and choosing an appropriate metric can be daunting, although important (Nakagawa and Cuthill, 2007). Hedge's *g* (Hedges, 1981; Cohen, 1988) is one such metric that has become popular in some fields. It is essentially a difference in means between the control and treatment states standardized to the deviation and weighted based on sample size (Borenstein et al., 2009). The Hedge's *g* metric therefore places more weight on data associated with smaller error. However, others (Osenberg et al., 1997, 1999) have raised concern over such error-based weighted metrics and have encouraged the use of metrics that are more relevant to the focal question. One example is *ln RR* (Hedges et al., 1999), which is calculated as the natural log-transformed ratio of the treatment and control mean values. Sample size and error are irrelevant to the calculation of this metric, thus allowing for the inclusion of data from studies that fail to provide data on sample size and error. For this metric, effect sizes greater than zero represent an increase relative to the control state (e.g., an *ln RR* of 1 equates to a ~3 fold increase), and those less than zero a decrease relative to the control state. Additionally, metrics can incorporate relevant temporal or spatial scales of the experiment (Osenberg et al., 1997), for example (*ln RR*)/time, which allows the incorporation of biologically important characteristics of the experiment. It is important to note the difference between an effect size metric and an effect size: an effect size metric is a statistical transformation that is applied to the raw data to describe (in this case) the difference between control and experimental states (e.g., *ln RR*) and an effect size is the numerical outcome of that statistical transformation.

In this meta-analysis, three different metrics were calculated for comparison: Hedge's *g*, *ln RR*, and (*ln RR*)/*t*. For calculation of Hedge's *g*, studies had to also include a variance component and sample sizes for the control and treatment means. Statistics associated with Hedge's *g* corresponding to heterogeneity between and within studies (*Q*, τ^2 , and I^2) were also calculated (Borenstein et al., 2009). Comprehensive Meta Analysis software (CMA) was used for all calculations of Hedge's *g* and associated statistics, while the R statistical software environment was used for comparing *ln RR*-based metrics between different types of studies.

2.3. Investigating characteristics of the studies

One purpose of a meta-analysis is to summarize effects across diverse studies to provide a general answer to whether or not a

manipulation influences a response (e.g., How much does the expression of a certain gene increase or decrease, or does it remain constant during salinity transfer?). However, the variation typically found within and between studies can shed light on other interesting patterns as well. In this context, several experimental factors that may impact salinity-induced gene expression were investigated. These included the broad taxonomic group and species studied and the time point after transfer when expression was measured. Characteristics of the salinity transfer were also noted, including: general direction of salinity transfer (i.e., from a higher to lower salinity or vice versa), absolute range of the transfer, and if the transfer involved a change in physiological state, such as from an osmoconforming to osmoregulating strategy (or vice versa). To characterize the latter types of transfers, additional studies (Appendix A) were consulted to determine over which salinities animals acted as osmoconformers vs. regulators (Fig. 1). Targeted specific isoforms or subunits of the genes were also recorded, when possible. In crustaceans, different pairs of gills were often investigated, as individual gills can be osmoregulatory vs. respiratory in function (for reviews, see Taylor and Taylor, 1992; Freire et al., 2008), so it was noted which gills were examined if possible (these were later combined into anterior vs. posterior gills). Methodological details were also recorded for each study including: whether expression levels were normalized to a “control” gene and if so which control gene was used, if the control state consisted of a sham transfer or a pre-transfer condition, and whether the salinity transfer was conducted in a controlled laboratory setting or if expression was measured in wild-caught animals that were inferred to have undergone a salinity transfer. It should be noted that many of these characteristics have often been explored in individual studies, and that meta-analysis is one way to quantitatively summarize these comparisons.

3. Results

3.1. The data set

All data used in our study have been made publically available in the online Supplemental material (Appendix A) in an attempt to promote transparency and encourage additional analyses with our dataset. Based on the literature search, 59 published studies were chosen for inclusion in the meta-analysis. Many others were considered but not included due to our earlier described acceptance criteria. Of the included papers, 45 targeted NKA, while NKCC (19), CA (11), CFTR (10), and HAT (10) were examined in fewer papers. Effect sizes (comparison of

expression between control and treatment states) also followed this trend, with 563 effect sizes calculated (using only a single metric; $\ln RR$) for NKA, 98 for NKCC, 104 for CA, 63 for CFTR, and 59 for HAT. Some studies only had a single calculable effect size, while Jayasundara et al. (2007) had the most of any paper examined: NKA isoforms at six different time points after both 35‰ to 45‰ and 35‰ to 10‰ transfers for two different years in five different pairs of gills were examined, amounting to a total of 200 effect sizes calculated. Analyses were performed again while excluding this paper to determine if it skewed our results. Including or excluding this study showed no significant influence on the overall effect size patterns (Fig. A.1) so data collected from Jayasundara et al. (2007) were included in our meta-analysis. A total of 29 different species were included in our meta-analysis, and expression was measured between two hours and 180 days after transfer. The papers examined crustaceans ($n=15$ papers), teleosts ($n=42$), or elasmobranchs ($n=2$), however the number of effect sizes were similar between crustaceans (50.5%) and teleosts (49.5%), because expression was often measured in multiple gills in crustaceans compared to a single measurement in teleosts. Many studies investigated the response of different isoforms, and control genes were generally used in studies investigating teleosts but not in those investigating crustaceans.

3.2. Effect metric performance and summary effects

Although all three effect metrics are presented in Appendix A for comparison, only results associated with $\ln RR$ are discussed here because: 1) data were usually most normally distributed when using this metric (Fig. 2), 2) results were generally similar between Hedge's g and $\ln RR$, and 3) elevated effect size variation associated with $(\ln RR)/t$ hindered our ability to identify clear trends in the dataset. When all effect sizes were grouped together, expression of all genes except CFTR significantly increased after salinity transfer, as shown by significant, positive $\ln RR$ summary effects (Fig. 3A). However, there was a large amount of heterogeneity in the studies for all genes, which indicates that generating a single summary effect may be misleading. This heterogeneity was investigated by partitioning the data into various groups and determining if there were significant differences in expression values between groups. Although all comparisons were performed for NKA, only a fraction of these comparisons were performed for the other genes due to low sample sizes. For example, CFTR expression was only examined in teleosts, so CFTR expression could not be compared between taxonomic groups.

In other cases, confounding effects existed for certain comparisons. For example, because studies of teleosts often used a control gene while those for crustaceans did not, these two characteristics cannot be teased apart. For NKA, the confounding effects were alleviated by portioning the data into smaller data sets (e.g., only those studies involving specific taxa, types of transfers, or tissues). When these smaller data sets were generated for the other genes, comparisons often became impossible or confined to single studies (i.e., not a meta-analysis) because of the reduced sample sizes. In all analyses, efforts were made to ensure multiple studies were compared, but to minimize confounding effects. Finally, publication bias was not an issue in the literature as the fail-safe number (the number of unpublished effect sizes needed to offset the summary effect of the meta-analysis) was calculated as more than 2000 for all genes (except CFTR; fail-safe numbers are not calculable when there is a non-significant summary effect). This suggests there is not a bias to publish studies showing significant changes in gene expression vs. those showing no significant change.

3.3. Factors contributing to heterogeneity between studies

The characteristics of the taxa being studied contributed to differences in gene expression between studies. Crustaceans tended to show the greatest increases in gene expression, while teleosts

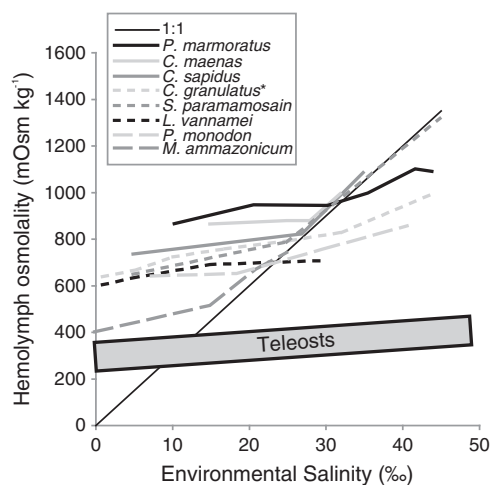


Fig. 1. Hemolymph osmolality of taxa analyzed in this study acclimated to various environmental salinities. Data are only presented for environmental salinities analyzed in this study. References used to gather this data are in Appendix A. *Note that *Chasmagnathus granulatus* has been renamed to *Neohelice granulata* (Sakai et al., 2006).

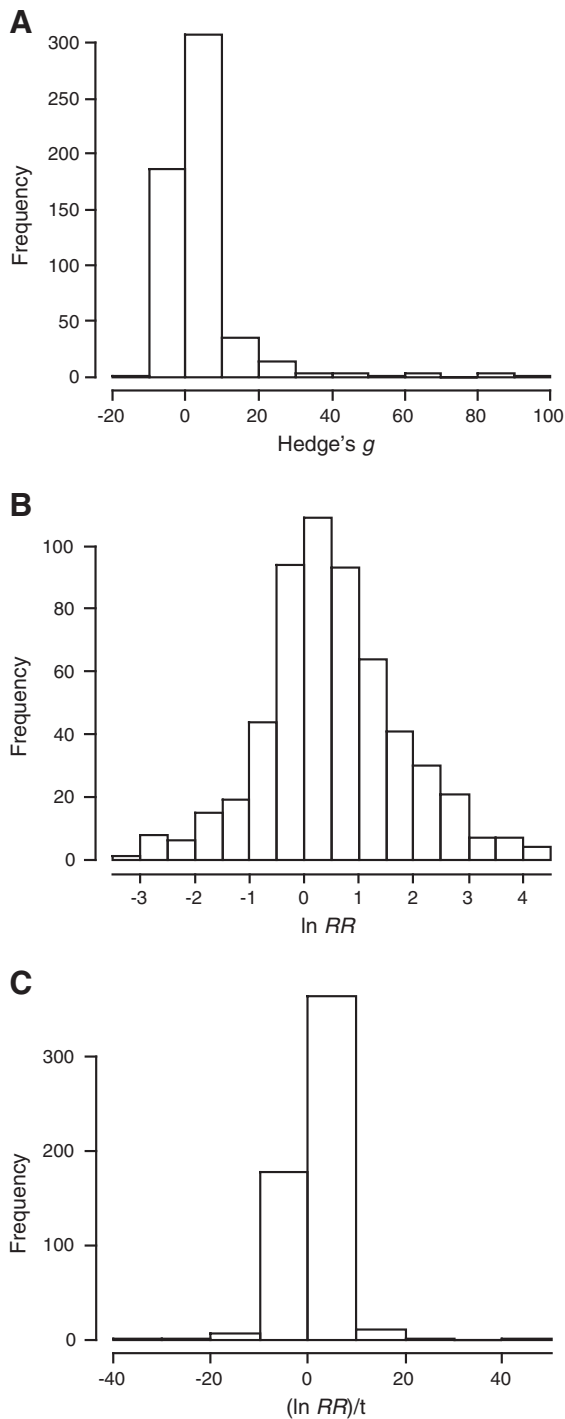


Fig. 2. Frequency distributions of the three effect metrics calculated for Na^+/K^+ -ATPase. (A) Hedge's g (Hedges, 1981; Cohen, 1988). (B) Natural log of the response ratio ($\ln RR$; Hedges et al., 1999). (C) $(\ln RR)/t$ (Osenberg et al., 1997). Results were similar for the other genes examined.

sometimes showed no significant change from control states (Fig. 3A). Additionally, for crustaceans, posterior gills tended to show greater increases in CA expression compared to anterior gills during salinity change (Fig. 3B). However, for NKA this trend was not significant ($P=0.598$). Finally, there was a large amount of variation between the different species studied (Fig. 4A). For teleosts, Salmoniforms tended to not change NKA expression during salinity transfer, while other teleosts had elevated NKA levels (Fig. 4B). Comparable results for CFTR (i.e., expression did not increase or decrease) were seen between both groups (Fig. 4B). Finally, intraspecific variation

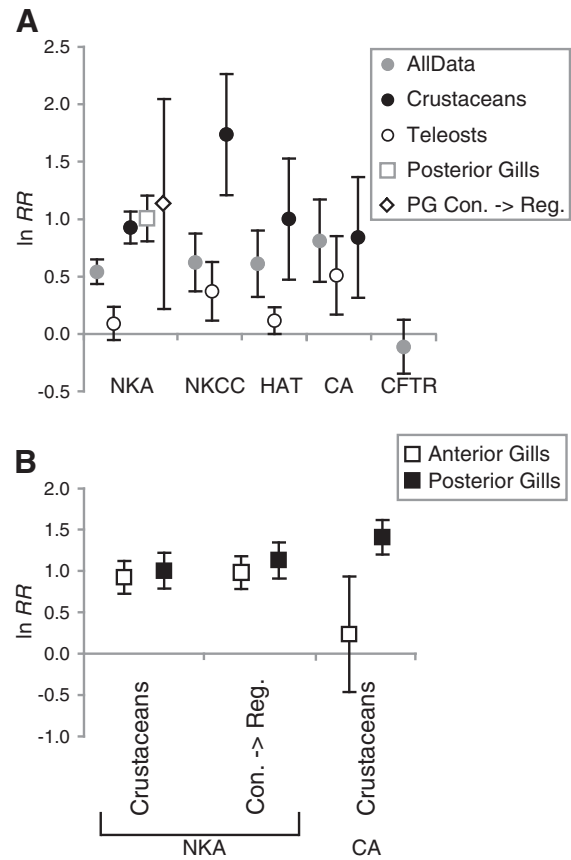


Fig. 3. Effect of taxa and tissue on expression of genes during salinity transfer. (A) $\ln RR$ ($\pm 95\%$ C.I.) for Na^+/K^+ -ATPase (NKA), $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC), carbonic anhydrase (CA), cystic fibrosis transmembrane regulator (CFTR), and H^+ -ATPase (HAT) for all data, crustaceans only, teleosts only, posterior gills of crustaceans only, or posterior gills of crustaceans undergoing an osmoconformer to osmoregulator switch (PG Con. \rightarrow Reg.). (B) Effect of tissue type on expression of NKA and CA in crustaceans, or only in crustaceans undergoing an osmoconformer to osmoregulator switch (Con. \rightarrow Reg.).

in NKA expression was able to be assessed for two teleosts, although there were no significant differences in changes between populations (Fig. 4C).

The characteristics of the salinity transfer also had an effect on gene expression. In all NKA datasets and for CA, transfers from a higher salinity to a lower salinity induced a higher increase in expression than transfers from lower to higher salinities (Fig. 5A). In NKCC and CFTR the opposite was true, and for HAT the direction of the transfer was not significant ($P=0.075$; Fig. 5A). Whether the transfer involved a switch in osmoregulation strategy also influenced gene expression. However, this could only be assessed accurately for expression of NKA and CA in crustaceans, as teleosts often acted as osmoregulators (although hyper- vs. hyporegulation was not considered) at all salinities investigated (Fig. 1) and other genes did not have sufficient samples for any meaningful comparisons. When crustaceans were transferred from salinities in which they were osmoconformers to salinities in which they were osmoregulators, expression of NKA and CA were elevated more than in other types of transfers (Fig. 5B). For CA this was most pronounced in the posterior gills (Fig. 5B). The absolute range of the transfer (e.g., a change of 10‰ vs. a change of 32‰) did not appear to result in significant differences in gene expression induction for the overall NKA, HAT, or NKCC datasets, but greater transfer ranges tended to result in greater increases in expression for crustacean NKA datasets and CFTR (Fig. 5C).

The duration of the salinity transfer also had a profound effect on gene expression. In many cases, gene expression peaked between 1

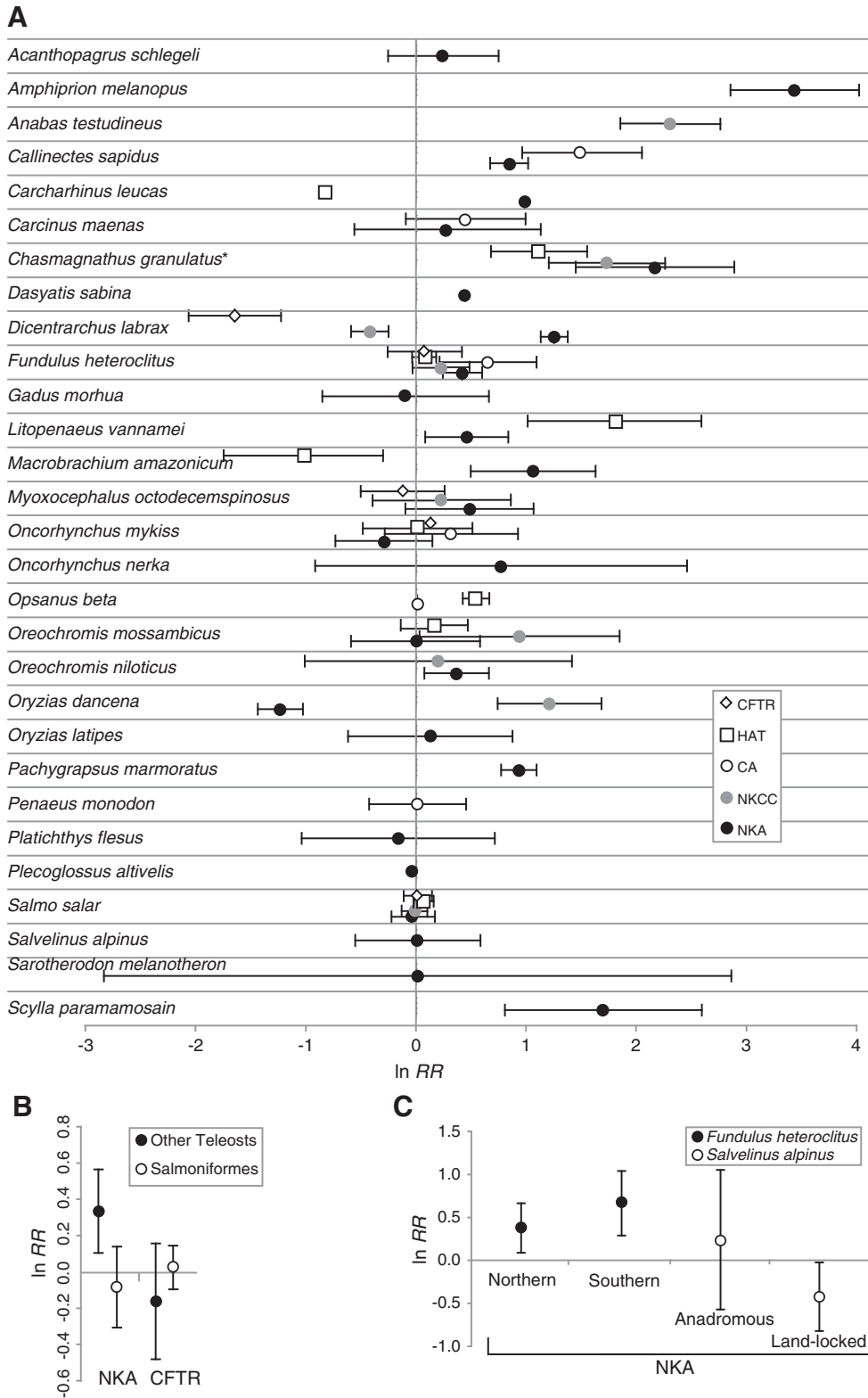


Fig. 4. Inter- and intraspecific variation in ln RR (\pm 95% C.I.) for genes examined in this study. (A) Effect of species. (B) Fishes in Salmoniformes had no significant NKA induction after salinity transfer, while those in other orders did, although both groups had similar changes in CFTR. (C) Intraspecific variation in NKA induction was not significant. Abbreviations follow Fig. 3. *Note that *Chasmagnathus granulatus* has been renamed to *Neohelice granulata* (Sakai et al., 2006).

and 3 days post transfer and then leveled off to control state levels by 2–3 weeks post transfer (Fig. 6A). However, notable exceptions to this trend include: 1) the posterior gills of crustaceans transferred from a salinity in which the organism is an osmoconformer to one in which it is an osmoregulator still showed elevated NKA expression at the latest

time points, 2) up-regulation of HAT expression appears at the earliest time points, and 3) CFTR expression did not vary with time. If NKA expression across time is examined in detail (Fig. 6B), a clear peak is present at 1–2 days post-transfer, and although other peaks are also present, they are often for time points with low sample sizes.

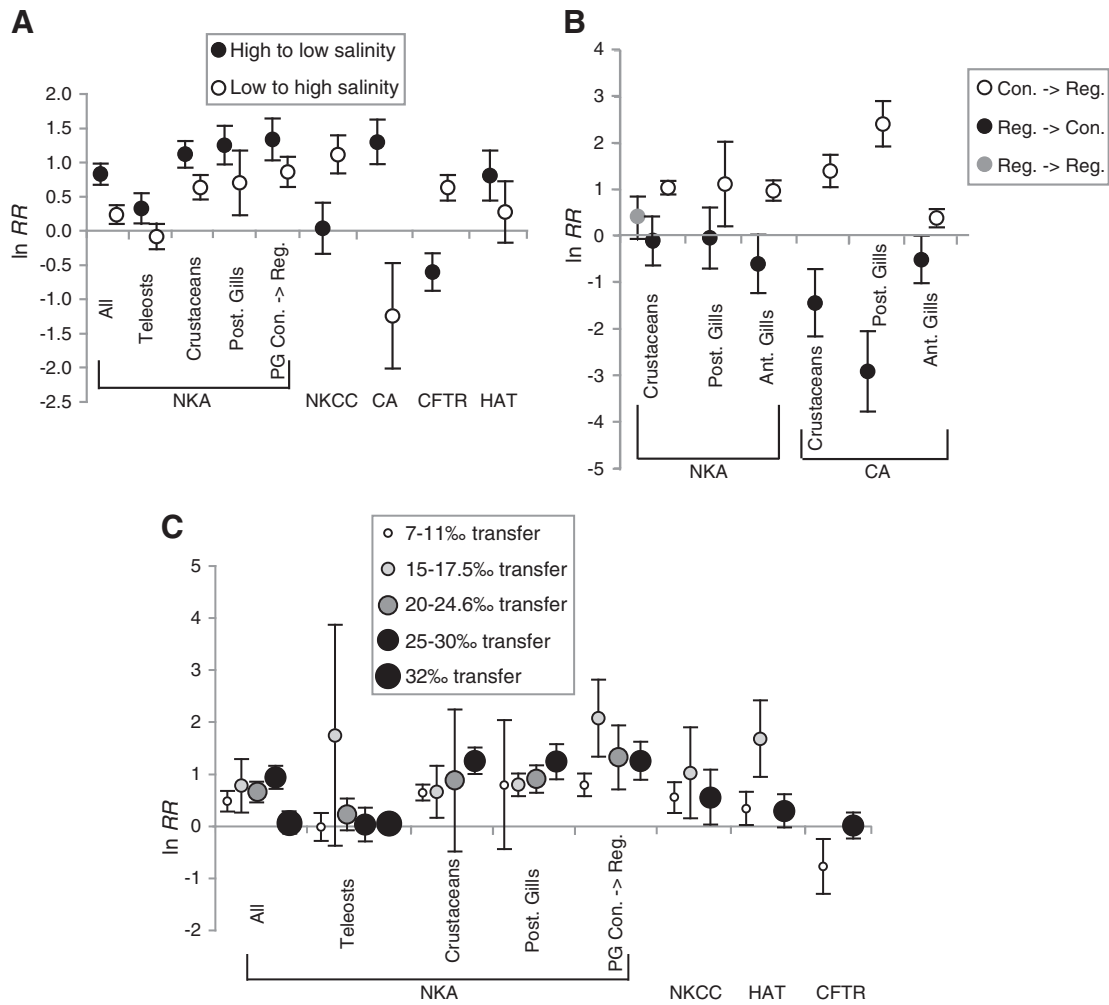


Fig. 5. Effect of type of salinity transfer on gene expression. (A) Transfers from higher to lower salinities produced greater increases in NKA and CA, while the opposite was true for CFTR and NKCC, and no difference was seen for HAT. (B) Transfers involving an osmoconformer to osmoregulator switch caused the highest levels of NKA and CA expression in crustaceans. (C) Transfers involving the most dissimilar salinities produced the highest levels of gene expression in NKA and CFTR. Abbreviations and error bars follow Fig. 3.

In some cases, differential expression of isoforms or subunits of the same gene was able to be assessed. NKA α and NKA β tended to have similar levels of expression (Fig. 7), although this may be because of the low sample sizes and high variation associated with NKA β . In teleosts, NKA α 1 and NKA α 3 had similar expression levels; however, NKA α 1b was up-regulated in response to salinity transfer, while NKA α 1a and NKA α 1c were not (Fig. 7). In crustaceans, two isoforms of branchial CA were compared: CAc (cytoplasmic) and CAg (membrane-associated). CAc is thought to be responsible for ion uptake, while CAg is involved in respiration/CO₂ excretion (Henry, 1988a,b; Henry et al., 2003). CAc was up-regulated in response to salinity transfer, while CAg was not; however, the difference between isoforms was only significant in posterior gills (Fig. 7). In anterior gills, the same general trend was found, although the difference between the isoforms was not significant ($P=0.239$, Fig. 7). Finally, HAT subunit a was up-regulated more than subunit b in response to salinity transfer (Fig. 7).

There were also differences in qPCR methodology used between studies. Some studies (47.8% of effect sizes) normalized expression to a control gene not related to osmoregulation, whereas others did not. Although studies using a control gene tended to show a lower level of gene expression when all data were considered, this is confounded by the fact that studies of teleosts tended to use a control gene, while those of crustaceans did not. When this was accounted

for, NKA expression levels within teleosts and crustaceans were the same between studies using and not using control genes (Fig. 8A), suggesting that results are not influenced by normalizing or not normalizing to a control gene. Similarly, there was not a difference between studies that expressed gene transfer change relative to a pre-transfer condition and those relative to a sham transfer measured at the same time point when taxonomic group was accounted for (Fig. 8B), suggesting calculating gene expression relative to a sham vs. pre-transfer yields similar results. Finally, for NKA expression in teleosts, it did not matter if the experiment was performed in a laboratory setting, or if a salinity transfer was inferred from environmental samples (Fig. 8C). However, most studies examined (245/253) were laboratory-based.

4. Discussion

4.1. Generalizations about the entire data set

Presented here for the first time is a quantitative summary of the effects of salinity transfer on expression of genes in euryhaline animals. This analysis summarizes 10 years of qPCR studies and provides a framework for future studies, whether they are focused on single genes or are genomics-based. Most published studies examined changes in gene expression for either crustaceans or teleosts, while two studies examined

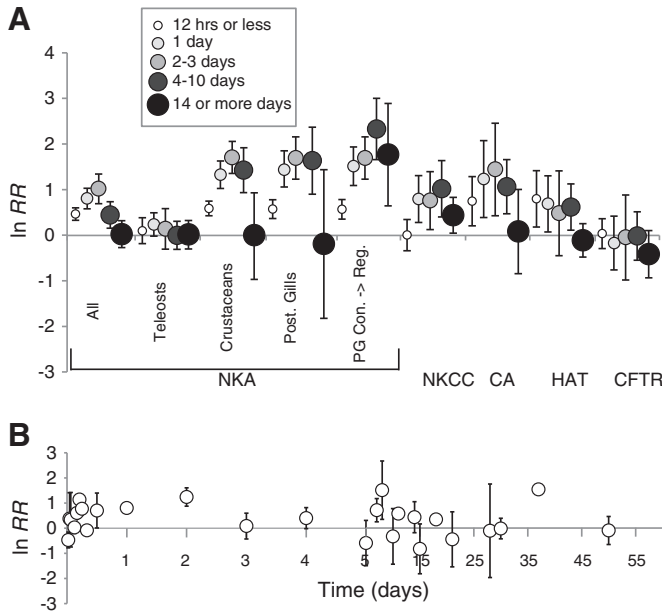


Fig. 6. Effect of duration of salinity transfer on gene expression. (A) Expression tended to peak at 1–3 days post-transfer for NKA, NKCC, and CA, but was highest immediately following transfer for HAT. (B) Detailed examination of NKA expression in response to duration of transfer for all data shows a peak at 1–2 days post-transfer, but other peaks also exist. Abbreviations and error bars follow Fig. 3.

elasmobranchs (Choe et al., 2005; Reilly et al., 2011). Although many other taxa are euryhaline, studies of gene expression during salinity transfer in these taxa were either focused on general stress proteins such as heat shock proteins and antioxidants (López-Legentil et al., 2008; De Zoysa et al., 2009; An et al., 2010) or used genomics-based methods like microarrays (Gracey et al., 2008; Lockwood and Somero, 2011). There were many other studies that were also examined initially, but later discarded due to: 1) use of genomics-based methods (e.g. Pinto et al., 2010; Towle et al., 2011), 2) use of semi-quantitative gel-based methods (e.g., Jensen et al., 1998; Mackie et al., 2005), and 3) unclear salinity transfer conditions (e.g., parts of Nilsen et al., 2007; Fan and Li, 2010).

It is not surprising that the genes examined are up-regulated during salinity transfer, as they have been implicated in salinity change response in many euryhaline animals for decades (Skou, 1965; Forrest et al., 1973; Frizzell et al., 1979; Henry and Cameron, 1982, 1983; Lin and Randall, 1991; Singer et al., 1998). A more interesting finding was that based on all papers that met the inclusion criteria, only CFTR expression was not influenced by salinity change, while NKA, NKCC, CA, and HAT expression were up-regulated, despite the vast heterogeneity between studies. It is important to note that this finding can only be generalized to the species and studies examined, and that down-regulation of these genes should be expected in some scenarios (e.g., crustaceans transferred from low to high salinities; Jillette et al., 2011). However, the studies investigated most likely tended to perform experiments that would result in up-regulation, and studies that would be expected to result in no change or a down-regulation should be targeted in the future.

4.2. Heterogeneity between studies

Grouping all studies together to “see the forest for the trees” is one advantage of a meta-analysis, but another advantage is to examine smaller groups of studies and compare them to the overall analysis or each other. Identifying the heterogeneity in a set of studies is one of the strengths of meta-analysis and allows researchers to make biological sense of why similar studies get different results. Because of the wealth of data for NKA, this was possible for many cases, although for other genes fewer meaningful comparisons could be made. For

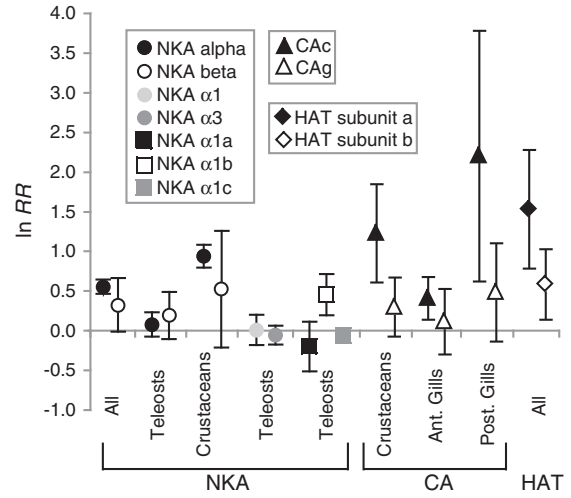


Fig. 7. Differential expression of isoforms and subunits. Only NKA α 1b shows increased expression in teleosts following transfer. CAC shows a higher level of expression than CAG following transfer. HAT subunit a also shows a higher level of expression than subunit b. Abbreviations and error bars follow Fig. 3.

example, several studies examined NKA expression in both anterior and posterior gills of crustaceans, but only a single study (Luquet et al., 2005) examined NKCC expression in different sets of gills. Therefore, a meaningful comparison could be made for NKA, but for NKCC, an analysis would just restate the results of Luquet et al. (2005).

Crustaceans likely had overall greater increases in gene expression than teleosts (Fig. 3A) because crustaceans often act as osmoconformers at higher salinities, but as osmoregulators at lower salinities, whereas teleosts are generally strong osmoregulators across a wide salinity range (Fig. 1). Therefore, salinity transfer can often entail a change in osmoregulatory strategy in crustaceans, but not in teleosts. However, one study (Gilmour et al., 2012) specifically transferred trout from seawater (SW) to iso-tonic conditions, but reported an increase in expression, suggesting transfer to iso-tonic conditions may be enough to initiate a SW to freshwater (FW) response. Gill specificity has been long-implicated in crustacean osmoregulation, and different species show differences in which gills act as osmoregulatory organs (Copeland and Fitzjarrell, 1968; Finol and Croghan, 1983; Cioffi, 1984; Towle, 1984; Wheatly and Henry, 1987; Compere et al., 1989; Dickson et al., 1991; Genovese et al., 2004; Freire et al., 2008). Posterior gills tended to have greater increases in CA expression than anterior gills for the taxa studied here (Fig. 3B), which is typical of the most-commonly studied species (i.e., decapod brachyurans). This general trend was also seen for NKA expression, but was only significant when Hedge’s g was used, suggesting studies that saw a difference between gills may have had greater sample sizes or less variation.

The wide variation shown between different studies examined is indicative of the fact that many of these general trends may not apply to all species. Because fishes in Salmoniformes had more studies and effect sizes than those in other orders, they were grouped together to show that salmoniforms generally do not alter NKA expression during transfer, but that the other species studied did increase NKA expression. This may be because salmoniforms generally only undergo anadromous (seawater to freshwater) migratory salinity changes based on life-history, while the other species studied are truly euryhaline and undergo salinity changes more frequently due to the environments they inhabit. For example, the most well studied non-salmoniform, the mummichog (*Fundulus heteroclitus*), inhabits coastal tidal creeks where salinity change may occur daily (Griffith, 1974). Therefore, different osmoregulatory mechanisms may exist for these two ecologically distinct groups. However, even salmoniforms increased NKA α 1b

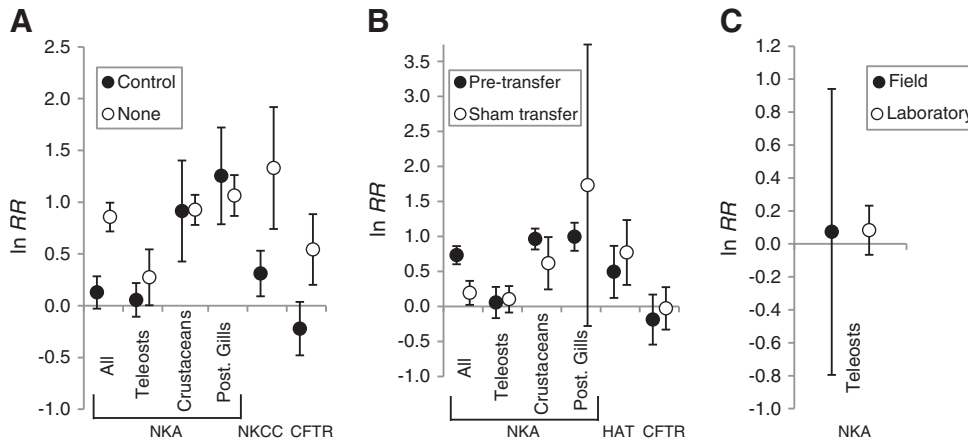


Fig. 8. Effect of methodological variables on gene expression following salinity transfer. (A) Using a control gene tended not to influence NKA expression when teleosts and crustaceans were analyzed independently. (B) Similarly, whether salinity-induced expression was compared to a sham transfer or pre-transfer condition did not influence expression values when taxonomic group was accounted for. (C) Although few studies examined NKA expression in a field-based setting, they were not different than those in laboratory settings for teleosts. Abbreviations and error bars follow Fig. 3.

expression after transfer (Fig. 7), showing that different isoforms can have different osmoregulatory roles. In fishes, NKA isoforms have different sequence characteristics near the N-terminus, suggesting differing responses to protein kinase regulation (Efendiev et al., 2000; Pierre et al., 2002; Richards et al., 2003), and providing a reason why different isoforms are up-regulated in response to transfer. Similarly, in crustaceans, CAC was significantly up-regulated after transfer, but CAG was not. This mirrors the roles of the two isoforms, as CAC is thought to be osmoregulatory, while CAG is respiratory (Henry and Cameron, 1983; Henry, 1988a,b; Serrano and Henry, 2008). Finally, for HAT, the finding that subunit a appears to be up-regulated more than subunit b during transfer has not been specifically explored previously. Meta-analysis as used here may therefore be useful for determining which isoforms to target in salinity transfer experiments of unexplored species.

The characteristics of the salinity transfer examined greatly affected the degree of gene expression change. Generally, the largest increases in NKA, NKCC, and CA expression were seen 1–3 days following salinity transfers from high to low salinities, when transfer represented a switch from animals acting as osmoconformers to osmoregulators. However, for NKA and CA specifically, a significant increase in expression was detected earlier — as little as 1 h after transfer (Faleiros et al., 2010). Adjusting levels of gene expression can be a rapid response to salinity, and changes in expression can occur before changes in protein activity, as has been well-documented for CA (Henry et al., 2003; Serrano et al., 2007; Serrano and Henry, 2008; Jillette et al., 2011). Therefore, future salinity transfer experiments should measure expression after acute, not chronic transfer. For most NKA datasets and other genes where expression was elevated, gene expression levels did not return to control levels until at least 14 days after transfer. However, when crustaceans were transferred from conforming to regulating salinities, their posterior gills still showed increased NKA levels at the longest time points measured (up to 28 days in Serrano et al., 2007). This suggests that chronically acclimated animals should be maintained in control salinities for several weeks before assessing control levels of gene expression (as suggested by Jillette et al., 2011).

Finally, methodological differences among the studies accounted for little of the observed heterogeneity. Generally, studies of fishes normalized gene expression to a control gene and compared experimental expression levels to individuals undergoing a sham transfer. Contrarily, studies of crustaceans did not normalize to a control gene and compared expression levels to a pre-transfer measurement, although they often compared expression to a non-osmoregulating control tissue (e.g., the anterior gills). When teleosts and crustaceans are assessed separately, it is clear that these different methodologies did not result in different levels of gene expression. Although

normalization to a control gene and sham transfers are desirable, limitations often exist that force researchers to adopt less optimal methods (e.g., the unavailability of a well-characterized control gene, as is case for the crustacean *Callinectes sapidus*; Serrano et al., 2007). This meta-analysis suggests that these additional controls are not critical to adequately assess salinity-induced changes in gene expression. Only four studies were explored that measured expression in a field-based setting, but these did not show significantly different changes in gene expression than laboratory-based studies. Further studies are needed to determine if the salinity-induced changes in gene expression observed in the laboratory are also observed under natural salinity transfer conditions. For example, field-based measurements of CA protein activity from *C. sapidus* collected in a stream-fed estuary have suggested that the responses typically seen in the laboratory may not mirror responses in reality (Jillette et al., 2011).

4.3. Areas for future research

The current meta-analysis has highlighted several questions that could be addressed in future studies. The most obvious is to apply meta-analyses to similar types of studies investigating other genes or changes in gene expression due to other types of environmental stress. Additionally, only teleosts, crustaceans, and two elasmobranch taxa were examined in the studies included here. Expanding qPCR based studies of salinity-induced gene expression to molluscs and other taxonomic groups should be a primary interest. Within crustaceans, decapod brachyurans were examined in most studies, which should be expanded to include more diverse taxa (e.g., shrimps, crayfishes, and lobsters). Differential expression of isoforms was also mainly confined to studies of teleosts. Teleosts have more genomic resources currently available than crustaceans, thanks to several well-established, annotated genomes from model teleosts (e.g., *Danio rerio*, *Takifugu rubripes*, and *Gasterosteus aculeatus*). In contrast, although EST libraries and other genomic resources are becoming available for some crustaceans previously used in osmoregulatory studies, the first crustacean genome (*Daphnia pulex*) was only recently sequenced (Colbourne et al., 2011). This may explain why studies involving teleosts often take multiple isoforms into account, whereas those of crustaceans usually do not. Vertebrates also underwent two rounds of genome duplication early in their evolution (Dehal and Boore, 2005), and teleosts have undergone an additional round of genome duplication (Christoffels et al., 2004), meaning that teleosts may simply have more isoforms than crustaceans. Studies involving crustaceans often involved a change from iso-osmotic conditions to hyper- or hypo-regulating conditions, but only a single fish study (Gilmour et al., 2012) measured expression during a transfer

to an iso-osmotic salinity. Additionally, no studies examined transfers from a hypersaline condition to either seawater or freshwater. These types of transfers should be explored. The few studies that examined intraspecific variation in gene expression focused on teleosts. These studies should be expanded in general. Finally, few studies examined gene expression in wild-caught animals, although some were not included because a reasonable approximation of time since transfer could not be estimated (Lorin-Nebel et al., 2012). Interpreting gene expression levels in the wild, or using controlled field-based mesocosm experiments should be a priority to determine if lab-based results are applicable to the changes that actually happen during migrations or movements between different salinity regimes.

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