



Differential tissue regulation of *nrf2/keap1* crosstalk in response to *Aeromonas* infection in Nile tilapia: a comparative study

Zizy I. Elbialy¹ · Abdullah S. Salah^{2,3} · Ahmed Elsheshtawy^{1,3} · Nasema M. Elkatatny⁴ · Alamira Marzouk Fouad⁵ · Haitham G. Abo-Al-El⁶

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Abstract

The antioxidant and detoxification systems involve intricate pathways in which nuclear factor erythroid 2-related factor 2 (Nrf2) and Kelch-like ECH-associated protein 1 (Keap1) play pivotal roles. In the basal state, reactive oxygen species are generated and neutralized in a balanced manner. However, stressors can disrupt this equilibrium, resulting in oxidative stress and cellular damage. In this study, we analyzed the expression of *nrf2* and *keap1* in Nile tilapia (*Oreochromis niloticus*) under homeostasis and challenge with *Aeromonas hydrophila*. During homeostasis, the predominant expression of *nrf2* was observed in the liver, blood, muscle, gut, and gills, while *keap1* was highly expressed in the brain, liver, blood, spleen, eye, head kidney, and gills. After the challenge, the spleen demonstrated the highest *keap1* expression, while the liver displayed the highest *nrf2* levels among the tissues examined. Apparently, our findings suggest that the spleen may be susceptible to initial damage following infection, leading to the manifestation of the first lesion. This susceptibility could be attributed to the spleen's high expression of *keap1*, acting as a negative regulator of *nrf2*. Notably, a positive correlation was observed between *nrf2* and *keap1* expression in several tissues, with the strongest association observed in the blood, gills, and head kidney under both normal and inflammatory conditions. Our findings indicate that blood may serve as a crucial mediator of Nrf2/Keap1 signaling in tissues like the liver and gut during normal and inflammatory states. By shedding light on the altered expression and correlation of *nrf2* and *keap1* in various tissues, this study elucidates their potential connection to antioxidant and immune responses, as well as the pathological features of *A. hydrophila* infection.

Keywords Antioxidant defense · Gene expression · Immune response · Keap1 · NRF2 · Nrf2:Keap1 signaling

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Extended author information available on the last page of the article

Introduction

Oxidative stress plays a crucial role in the health of aquatic organisms and, consequently, in the aquaculture industry (Abdel-Mageid et al. 2020; Burgos-Aceves et al. 2021). Stressors can be both endogenous (e.g., cellular biochemical reactions) and exogenous (e.g., xenobiotics, pollutants, drugs, heavy metals, ionizing radiation, and disease agents). These elements can lead to the overproduction of reactive oxygen species (ROS) and electrophiles, overwhelming the cellular antioxidant capacity and causing oxidative stress (Abo-Al-Ela and Faggio 2021; Thannickal and Fanburg 2000). ROS can react with biomolecules like nucleic acids, proteins, and lipids, resulting in the generation of secondary electrophilic products called electrophiles (Li et al. 2016). These reactions ultimately lead to cell and tissue injury or damage.

Normally, when the microenvironment (whether intracellular or extracellular) of a cell changes, the cell responds to maintain its normal function. This response involves several steps: (1) a stimulus from either extracellular factor(s) (e.g., cytokines released by adjacent cells) or intracellular elements or stressors (e.g., high levels of ROS), (2) activation of the receptor, which then stimulates relevant cellular signaling pathways, and (3) certain intracellular signaling protein(s) called effector protein(s) delivering appropriate signals to intracellular targets (Li et al. 2016).

ROS act as mediators of inflammatory and immune responses, and these processes are closely interconnected (Li et al. 2016; Thannickal and Fanburg 2000). ROS trigger the production and release of inflammatory mediators, including cytokines. Excessive ROS production and inflammatory mediator release disrupt the normal immune state, leaving organisms vulnerable to pathogens and diseases (Marcos-López and Rodger 2020).

The nuclear factor erythroid 2-related factor 2 (Nrf2):INrf2 (Kelch-like ECH-associated protein 1; Keap1) signaling pathway is a cellular mechanism that senses oxidative and electrophilic stressors such as chemicals and radiation (Kaspar et al. 2009). Nrf2, a member of the cap'n collar family of basic region-leucine zipper transcription factors, acts as a major regulator of antioxidant genes and enhances ROS detoxification (Wang and Zhu 2019; Wu et al. 2017). Keap1, a component of the Cullin3-based ubiquitin E3 ligase, interacts with Nrf2, repressing its function and its dependent pathways (DeNicola et al. 2011). In the basal state, Nrf2 is constitutively expressed and maintained at a balanced level by binding to Keap1, which leads to its degradation. However, when organisms experience stress, Nrf2 becomes detached from Keap1, activated, and translocates to the nucleus to activate the expression of a wide range of genes. These genes encode antioxidant and detoxifying enzymes (e.g., superoxide dismutase, catalase, hemoxygenase-1, glutathione, and glutathione peroxidase), antiapoptotic proteins, proteasomes, and drug transporters, thus restoring the normal physiological state through their respective pathways (Niture et al. 2014; Ramsden and Gallagher 2016).

Fishes are consistently subjected to various stressors, including diseases. Among these stressors, bacterial infections are significant contributors to various disease conditions (Tartor et al. 2021; Van Doan et al. 2022). *Aeromonas hydrophila* is commonly recognized as an opportunistic pathogen, often acting as a secondary invader (Semwal et al. 2023). However, it has been observed to cause outbreaks (Marinho-Neto et al. 2019; Tartor et al. 2021). Consequently, it is crucial to enhance our understanding of the pathological pathways associated with these microorganisms.

Despite numerous studies on Nrf2:INrf2 (Keap1) signaling in various species, the functions of these proteins in fish remain poorly understood. Therefore, our study aimed

to investigate the gene expression of *nrf2* and *keap1* in different organs of Nile tilapia (*Oreochromis niloticus* L.), as they play key roles in the antioxidant and immune systems. We analyzed their expression levels both in the normal healthy state and following a bacterial challenge with *A. hydrophila*. Our goal was to explore the potential relationship between *nrf2*, *keap1*, and bacterial infection. In addition, we hypothesized that the various tissues would exhibit diverse levels of response and expression due to the close interaction between Nrf2 and Keap1. Hence, we investigated the potential impact of *keap1* on *nrf2* expression and explored any correlations between their expression levels in different tissues.

Materials and methods

Fish facility and rearing conditions

A total of 144 7-month-old male Nile tilapia (*Oreochromis niloticus* L.) weighing 86.3 ± 1.6 g were obtained from a local fish farm in the Kafrelsheikh governorate. The fish were transported to the Biotechnology Laboratory of the Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, and placed in 12 tanks with dimensions of $70 \times 50 \times 40$ cm and a capacity of 140 L.

Upon arrival, the fish were given a 2-week acclimation period during which they were fed commercial feed twice a day at 3.5% of their body weight to maintain optimal water quality parameters. The waste was siphoned daily, and approximately one-third of the aquarium water was changed day after day to maintain optimal water quality parameters. Aeration was provided to ensure normal levels of dissolved oxygen (6.8 ± 0.5 mg/L). The fish were reared under controlled conditions of 27 ± 0.5 °C and a pH of 7.8 ± 0.4 , and a 12-h light and 12-h dark cycle. Their health status was carefully monitored upon arrival and after the acclimatization period before conducting the bacterial challenge.

Bacterial preparation and challenge

The isolate underwent several characterization procedures. Morphological characterization involved conducting Gram stain and motility tests on semisolid media. Biochemical characterization was performed using the VITEK-compact microbial identification system. Subsequently, the isolate was sub-cultured on *Aeromonas* medium base (CM0833, Oxoid Ltd, UK). For molecular characterization, PCR was carried out using a universal bacterial primer. The resulting PCR product was purified and sequenced, followed by a BLASTn sequence similarity search against the GenBank database. Additionally, PCR was used to identify and sequence the *aerolysin-A toxin* gene, which serves as a pathogenicity marker for the isolate. To confirm the species, the purified *Aeromonas* spp. 16 sRNA PCR amplicon underwent restriction digestion, resulting in a single cut and two fragments (1039 and 462 bp). For more information, please refer to Elsheshtawy et al. (2019).

Before the experimental challenge, a pure culture of *A. hydrophila* was grown on trypticase soy agar (TSA) at 28 °C for 24 h. A colony of *A. hydrophila* was carefully selected and mixed with 10 mL of tryptic soy broth, and then incubated for 18 h using a shaking incubator. Once the OD₆₀₀ of this culture reached 0.6, ten-fold serial dilutions were prepared, and 1 mL of each dilution was spread over TSA plates. The plates were then incubated at 28 °C overnight. Three replicates of each dilution were plated on TSA. A dilution

that resulted in 30–300 colonies was chosen to calculate CFU/mL. The sixth dilution had an average of 92 colonies, equivalent to 9.2×10^7 CFU/mL, and was selected as it corresponded to the estimated $LD_{50-96\text{ h}}$.

Prior to the challenge, we conducted a preliminary estimation of $LD_{50-96\text{ h}}$, using bacterial concentrations of 10^6 (min), 5×10^6 , 10^7 , 5×10^7 , 10^8 , 5×10^8 , 10^9 , and 5×10^9 (max) CFU/mL. Regression analysis of the mortality curves indicated that the estimated $LD_{50-96\text{ h}}$ was 9.2×10^7 CFU/mL (Abdel-Tawwab and El-Araby 2021; Li et al. 2011; Lu et al. 2019; Mabrouk et al. 2022; Mu et al. 2017). For the challenge trial, we selected a sublethal dose of $1/10 LD_{50-96\text{ h}}$ (9.2×10^6 CFU/mL) (Abdel-Tawwab and El-Araby 2021).

For the bacterial challenge, a fresh broth culture of *A. hydrophila* was prepared as described earlier and incubated at 28 °C until it reached an OD_{600} of 0.6. Bacterial cultures were diluted to obtain a sublethal dose, specifically $1/10$ of the $LD_{50-96\text{ h}}$ dose (9.2×10^6 CFU/ml) (Abdel-Tawwab and El-Araby 2021). The bacterial cells were then pelleted by centrifugation at $1500 \times g$ using a cooling centrifuge at 4 °C. Afterward, they were washed once with PBS and resuspended in PBS to create the bacterial suspension.

After the acclimatization period, the fish were randomly divided into two groups (6 tanks/group): the challenged group and the control group. A suspension of *A. hydrophila* (9.2×10^6 CFU/mL) was prepared, and the challenged group was intraperitoneally injected with 0.2 mL of the bacterial suspension, while the control group received the same amount of sterile phosphate-buffered saline. Infection was confirmed by re-isolation of the inoculated bacteria from the challenged fish at the end of the challenge, and pathogenicity was confirmed through PCR amplification of the *A. hydrophila aerolysin-A toxin* gene (primers are provided in Table 1).

Sampling

At 24 h post-challenge, tissue samples were collected from both the challenged and non-challenged groups, with 12 fish per group. To euthanize the fish, tricaine methanesulfonate (MS-222) was administered at a concentration of 25 mg/L. Tissue samples including visceral adipose tissue, blood, brain, eye, fin, gills, heart, intestine, head kidney, liver, muscle, skin, spleen, stomach, and testes were collected and placed in Eppendorf tubes. The tubes were then stored at a temperature of $-80\text{ }^{\circ}\text{C}$ for further analysis.

Total RNA extraction, cDNA synthesis, and quantitative real-time PCR assay

Total RNA was extracted from 100 mg of the different samples (12 fish from each group) using TRIzol® (iNtRON Biotechnology, Inc., South Korea) according to the manufacturer's manual. The extracted RNA samples were then assessed for quality and quantity using a spectrophotometer (BioDrop µLITE®, Biochrom Ltd, Cambridge, UK) to ensure accurate analysis of extremely small samples with tremendous reproducibility. The integrity of the RNA was further evaluated through electrophoresis in a 1.5% agarose gel containing 0.5% ethidium bromide (Sigma, Germany), and observation was done under a UV transilluminator (Azure c200®).

For cDNA synthesis, 2 µg of total RNA was used with the HiSenScript cDNA synthesis kit (iNtRON Biotechnology). The synthesized cDNA served as a template for determining the relative expression of the *nrf2* and *keap1* genes using a Mic qPCR Cyclyer® (Bio Molecular Systems, Australia).

Table 1 Primers used for quantitative real-time PCR analysis

| Target gene | Primer sequence 5'-3' | NCBI accession number | Efficiency (%) | Efficiency R ² | Amplicon size (bp) | Annealing temperature | Reference |
|--------------------------|--|-----------------------|----------------|---------------------------|--------------------|-----------------------|--------------------|
| <i>nrf2</i> | F: CTGCCGTAAACGCAAGATGG R: ATCCGTTGACTGCTGAAGGG | XM_003447296.5 | 98 | 0.99 | 226 | 57 °C | Jia et al. (2019) |
| <i>keap1</i> | F: CTTCCGCCATCATGAACGAGC R: CACCAACTCCATACCGCACT | XM_003447926.4 | 97 | 0.99 | 181 | 57 °C | Jia et al. (2019) |
| <i>18S rRNA</i> | F: GGACACGGAAAGGATTGACAG R: GTTCGTTATCGGAATTAAACCAGAC | JF698683 | 96 | 0.98 | 111 | 65 °C | Yang et al. (2013) |
| <i>efl1a</i> | F: GCACGCTCTGCTGGCCTTT R: GCGCTCAATCTTCCATCCC | AB075952 | 94 | 0.99 | 250 | 66 °C | Yang et al. (2013) |
| <i>UBCE</i> | F: CTCTCAAATCAATGCCACTTCC R: CCCTGGTGGAGTTCTTTGT | XM_003460024 | 99 | 0.99 | 130 | 59 °C | Yang et al. (2013) |
| <i>aerolysin-A toxin</i> | F: TTGACCTCGGCCTTGAACTC R: GTGAAACCGAACTGGCCATC | KX138395.1 | – | – | 187 | 59 °C | This study |

efl1a elongation factor 1- α , *nrf2* nuclear factor erythroid 2, *keap1* Kelch-like ECH-associated protein 1, *UBCE* ubiquitin conjugating enzyme E2

The PCR mix consisted of 12.5 μL of 2X SensiFAST SYBR Green/no-ROX qPCR Master Mix (Bioline, USA), 2 μL of cDNA template, 1 μL of forward primer, 1 μL of reverse primer, and 8.5 μL of nuclease-free water. The reaction cycle was programmed as follows: an initial denaturation step at 95 $^{\circ}\text{C}$ for 5 min, followed by 40 cycles at 95 $^{\circ}\text{C}$ for 5 s, 57–66 $^{\circ}\text{C}$ (as specified in Table 1) for 20 s, and 72 $^{\circ}\text{C}$ for 10 s. A dissociation analysis step was also included. The gene-specific primers used are listed in Table 1.

To determine the amplification efficiency of each primer set, the slopes of the amplification curves obtained from mixed cDNA templates were calculated using the equation: $E = 10^{(-1/\text{slope})}$.

The C_t values of the target genes (*nrf2* or *keap1*) from healthy tissues were normalized against internal reference genes (i.e., ubiquitin conjugating enzyme E2 (*UBCE*), *18S rRNA*, and elongation factor 1-alpha (*ef1 α*)) (Yang et al. 2013) to obtain efficiency-corrected relative gene expression using the $E^{-\Delta C_t}$ method (Chini et al. 2007; Ganger et al. 2017; Moreno Abril et al. 2018; Pfaffl 2001), where the relative expression of the target genes in the challenged fish tissues was normalized against their expression in the same tissues prior to infection, based on the modified $E^{-\Delta\Delta C_t}$ (Chini et al. 2007; Ganger et al. 2017; Pfaffl 2001; Yuan et al. 2006). This approach accounts for correction of unequal amplification efficiencies and multiple reference genes (modified $2^{-\Delta\Delta C_t}$). The equation used for calculation was as follows:

$$\text{Relative expression of GOI (R)} = \frac{\text{geoMean}(E_{\text{REF}})^{C_{t,\text{challenged}}}}{(E_{\text{GOI}})^{C_{t,\text{challenged}}}} \div \frac{\text{geoMean}(E_{\text{REF}})^{C_{t,\text{healthy}}}}{(E_{\text{GOI}})^{C_{t,\text{healthy}}}}$$

where $\text{geoMean } E_{\text{REF}}$ represents the geometric mean of the efficiencies of the reference genes (internal housekeeping calibrators) in the examined tissue sample, E_{GOI} denotes the efficiency of the gene of interest (*keap1* or *nrf2*), $C_{t,\text{challenged}}$ represents the cycle threshold value of a gene in the tissues of the challenged fish, and $C_{t,\text{healthy}}$ represents the cycle threshold value in the healthy fish.

Statistical analysis

Prior to conducting statistical modeling, the data underwent normality testing and outlier detection using Shapiro–Wilk’s normality test and two-sided Grubbs’ test, respectively. To optimize data modeling and distinguish the overall variation in *nrf2* gene expression (continuous response variable) across different tissues (categorical predictor variable) with and without infection (categorical predictor variable), as well as its relation to the expression of the *keap1* gene (quantitative predictor), we employed analysis of covariance (ANCOVA) as a multiple regression model. To determine the best regression model to fit our data, four different models, each with different regression equations, were run using GraphPad Prism version 9.0® (GraphPad Software, Inc., San Diego, CA, USA). These models were compared using both the results obtained from the sum of square F-test ($p > 0.05$) and the Akaike information criterion (AIC). Based on this model comparison, the two-way interaction model was found to be the best fit for our data. The equation for this model is as follows: [$\text{nrf2} \sim \text{Intercept} + \text{Tissue} + \text{Infection} + \text{keap1} + \text{Tissue} : \text{Infection} + \text{Tissue} : \text{keap1} + \text{Infection} : \text{keap1}$]. The adipose tissue and non-infection status were considered the reference levels for the tissue variable and infection variable, respectively. The results of the regression model are presented in the corresponding graph.

To further examine the differential relative expression of each individual gene of interest in the examined tissues under healthy and challenged conditions, we performed one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Significant differences were determined at a p -value ≤ 0.05 . Additionally, to assess the association between the two studied genes (*nrf2* and *keap1*) in the same tissue, Pearson's correlations were estimated for the relative expression of the two genes in each tissue, separately for pre- and post-infection statuses. The correlation coefficient (r) and two-tailed p -value were reported on the correlation graph for each tissue. Data are presented as means \pm SEM.

Results

Expression pattern of *nrf2* and *keap1* during the normal health state

During the normal health state, gene expression analysis revealed a wide range of expression for *nrf2* and *keap1* in various tissues, as compared to the geometric mean of house-keeping genes ($2^{-\Delta Ct}$; Fig. 1). Among the tissues, the liver exhibited the highest expression

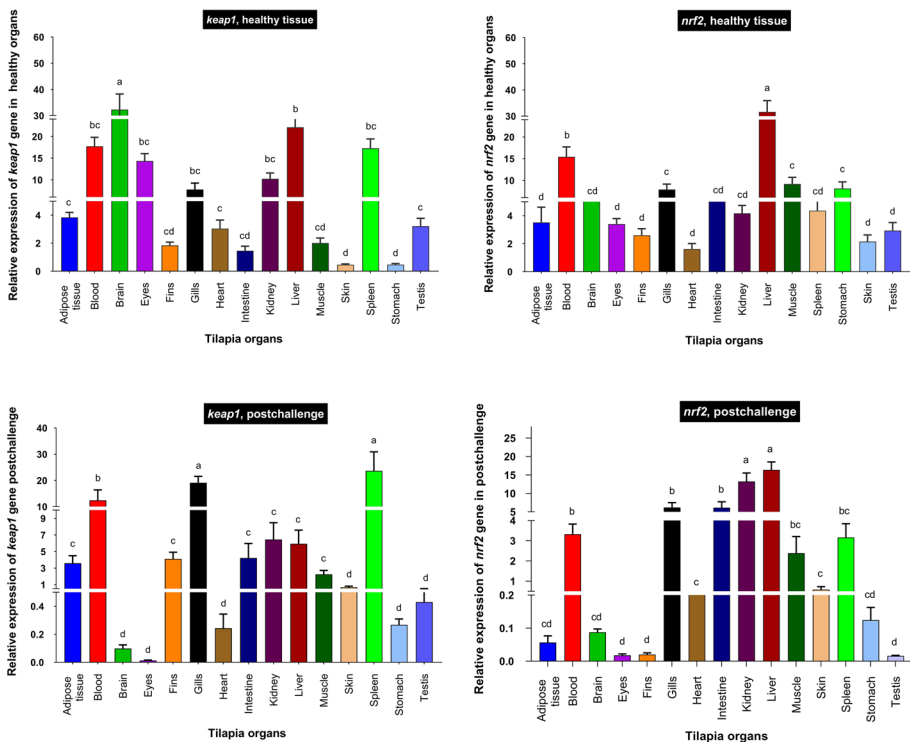


Fig. 1 Relative expression analysis of *nuclear factor erythroid 2-related factor 2* (*nrf2*) and *kelch-like ECH-associated protein-1* (*keap1*) in different tissues of Nile tilapia (*Oreochromis niloticus* L.) under normal conditions (healthy tissues; $2^{-\Delta Ct}$) or after challenge with *Aeromonas hydrophila* ($2^{-\Delta \Delta Ct}$). The data were analyzed using Brown-Forsythe one-way ANOVA followed by Tukey's multiple comparisons test. The data are expressed as means \pm SEM. Superscript letters indicate significant differences between tissues on the same graph ($p \leq 0.05$)

value of 31.6 for *nrf2*, followed by the blood and muscle with values of 15.4 and 9.2, respectively (Fig. 1).

On the other hand, the heart showed the lowest expression value of 1.6 for *nrf2* among the tissues with the lowest expression, namely the testes, fins, skin, and heart. However, there were no significant differences in *nrf2* expression among these tissues. The brain, intestine, spleen, and head kidney exhibited moderate expression of *nrf2*, ranging from 5.7 to 3.4 (Fig. 1).

In contrast, *keap1* displayed a different expression pattern among the examined tissues. The highest range of expression was observed between 32.3 and 10.2, with the brain exhibiting the highest expression, followed by the liver and blood with values of 22.1 and 17.7, respectively. The gills exhibited an expression value of 7.8, while the remaining tissues displayed a descending pattern of expression, reaching the lowest value of 0.5 in the stomach and skin (Fig. 1).

Expression pattern of *nrf2* and *keap1* postchallenge with *A. hydrophila*

After challenge with *A. hydrophila*, the liver and head kidney exhibited significant upregulation of *nrf2* expression, with fold changes of 16.36 and 13.22, respectively, compared to the control (Fig. 1). The gills and intestine showed approximately sixfold upregulation, while the blood, spleen, and muscle displayed increases of around threefold compared to the control. On the other hand, the skin, heart, stomach, brain, adipose tissue, eyes, fins, and testes showed downregulation with minor changes in *nrf2* expression compared to the control (Fig. 1).

In challenged tilapia, *keap1* demonstrated a different expression pattern compared to *nrf2*. The spleen exhibited a dramatic upregulation, while the head kidney and liver showed significant upregulation, although to a lesser extent than the spleen (Fig. 1). Moderate upregulation, ranging from 4.3- to 2.3-fold compared to the control, was observed in other tissues such as the intestine, fin, adipose tissue, and muscle. Conversely, the skin, testes, stomach, heart, brain, and eyes exhibited decreased *keap1* expression, particularly the eyes, when compared to the control (Fig. 1).

Correlation and ANCOVA and crosstalk between the expression of *nrf2* and *keap1*

In the pre-infection (control) state, correlation analysis of the normalized expression of the *nrf2* and *keap1* genes revealed a very strong correlation in the liver and gills, a strong correlation in the blood, eye, and spleen, and a fairly strong correlation in the head kidney. These correlations were positive and exhibited a linear pattern (Fig. 2; Additional File 1: Table S1). However, the correlation was weak or negligibly positive in the brain, heart, muscle, skin, and testis.

Upon challenge with *A. hydrophila*, the strongest positive linear correlation was observed in the blood, intestine, gills, and head kidney. A moderate positive correlation was seen in the liver, skin (linear), spleen, and fins (nonlinear). In the other studied tissues, the correlation was weak or negligible (Fig. 3; Additional File 1: Table S2).

Controlling for the effects of *keap1* transcript levels and infection, a significant difference in the expression of *nrf2* between tissues was observed (ANCOVA: $f_{14,343} = 73.62$, $p < 0.001$). Upregulation of *nrf2* was evident in the blood ($t = 3.79$, $p = 0.002$), gills ($t = 2.66$, $p = 0.008$), muscle ($t = 2.4$, $p = 0.016$), liver ($t = 8.46$, $p < 0.0001$), and stomach ($t = 2.54$, $p = 0.013$) compared to adipose tissue (Fig. 4; Additional File 1: Table S3). Furthermore, infection had a significant impact on the relative expression of *nrf2* (ANCOVA: $f_{1,343} = 86.3$, $p < 0.001$) when

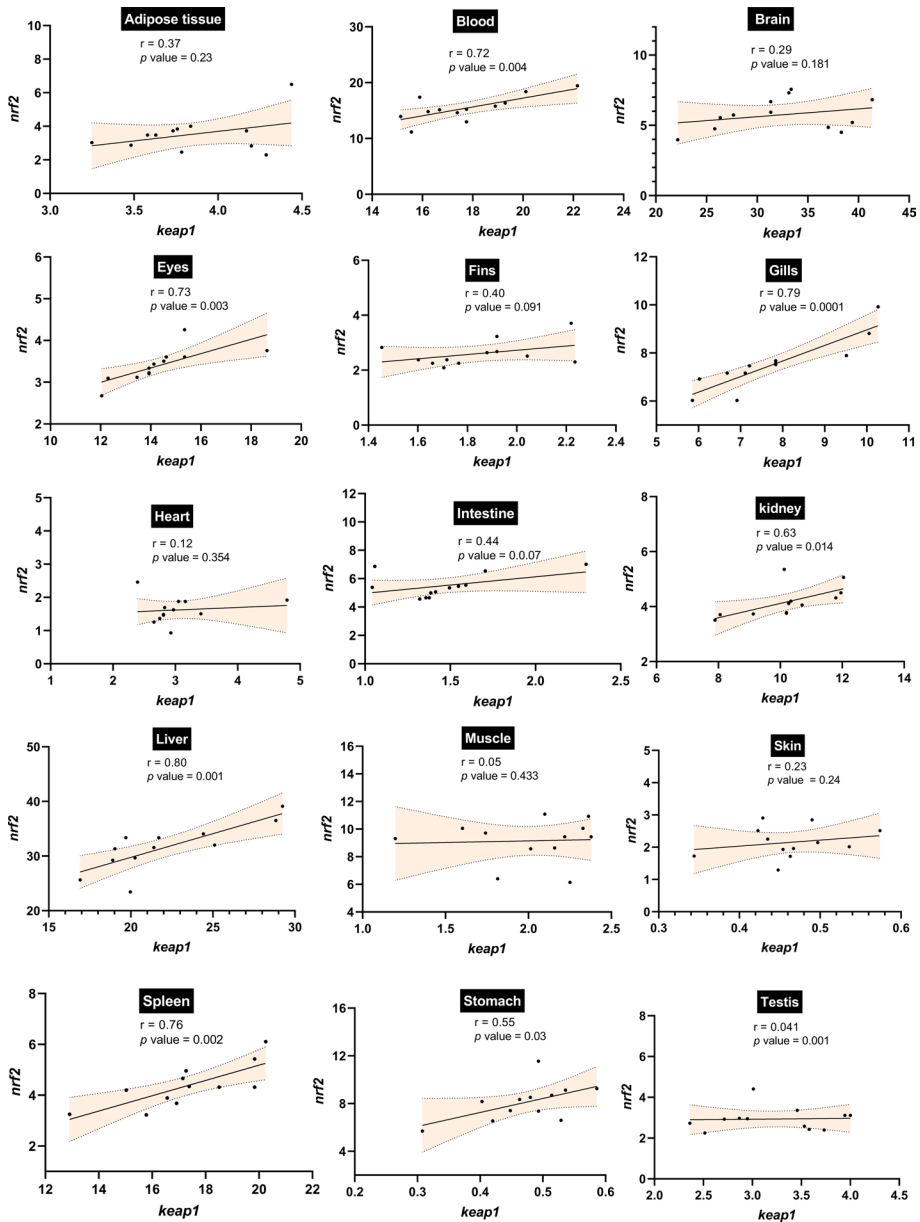


Fig. 2 Correlation of nuclear factor (erythroid-derived 2)-like 2 (*nrf2*) with kelch-like ECH-associated protein-1 (*keap1*) in the different tissues of Nile tilapia (*Oreochromis niloticus* L.) under normal conditions. Pearson's correlations were estimated between the relative expression of the two genes in each tissue. The correlation coefficient (r) and the p value (two-tailed significance) are presented on the graph for each tissue

controlling for tissue type and *keap1* transcript levels. The expression level of *keap1* significantly influenced the expression level of *nrf2* (ANCOVA: $f_{1,343} = 34.45$, $p < 0.001$) when controlling for the effects of tissue type and infection (Fig. 4; Additional File 1: Table S3).

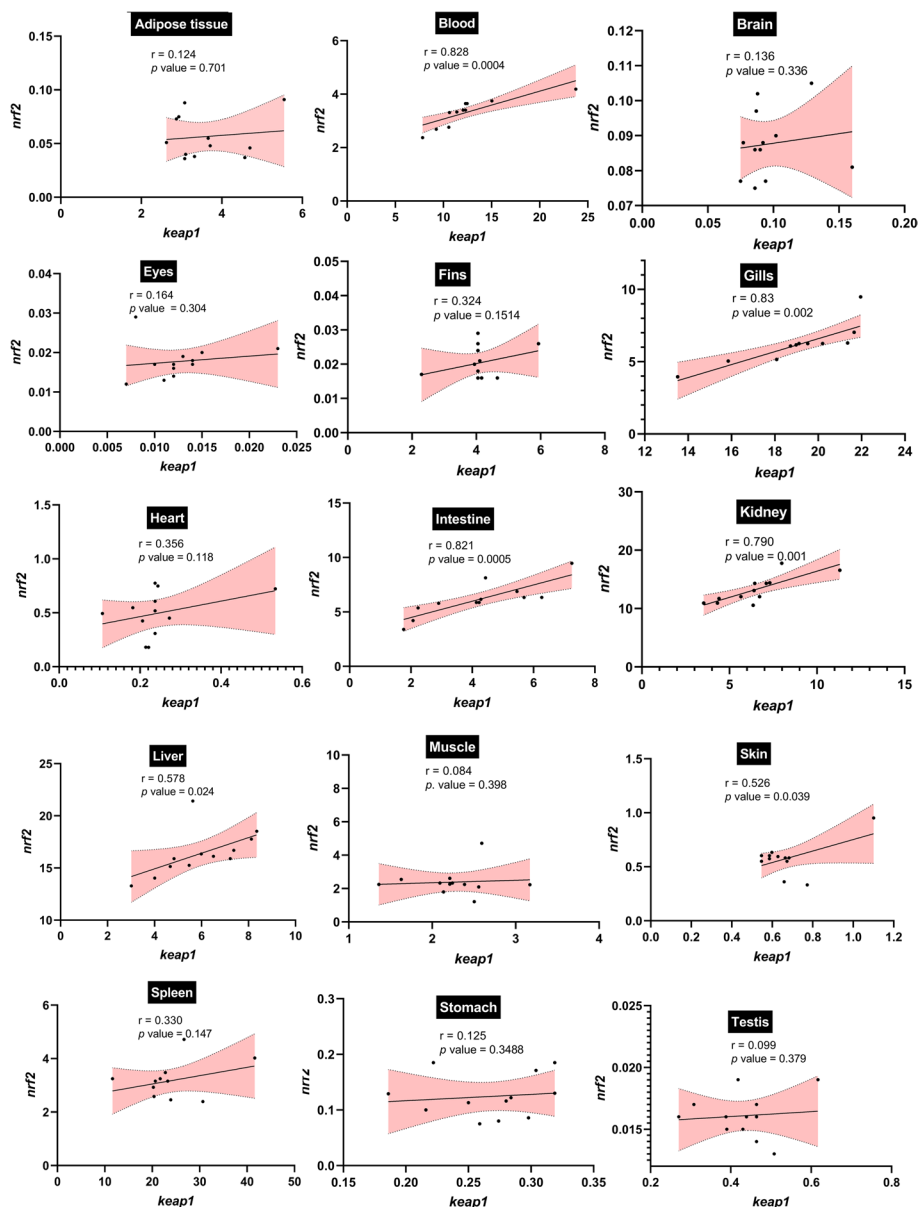


Fig. 3 Correlation of nuclear factor (erythroid-derived 2)-like 2 (*nrf2*) with kelch-like ECH-associated protein-1 (*keap1*) in different tissues of Nile tilapia (*Oreochromis niloticus* L.) challenged with *Aeromonas hydrophila*. Pearson's correlations were estimated between the relative expression of the two genes in each single tissue. The correlation coefficient (*r*) and the *p* value (two-tailed significance) are presented on the graph for each tissue

Discussion

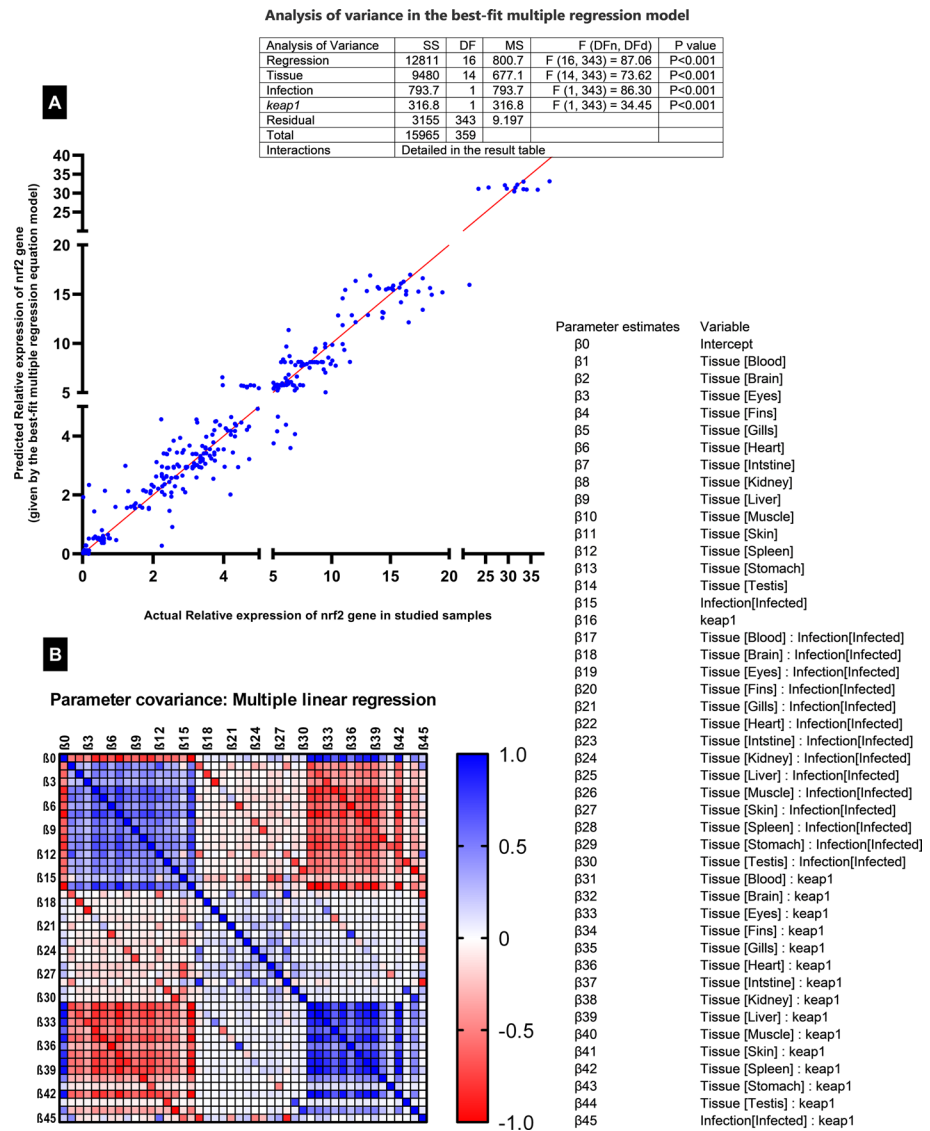


Fig. 4 Two-way ANCOVA model fitting the data, equation: [$nrf2 \sim \text{Intercept} + \text{Tissue} + \text{Infection} + \text{keap1} + \text{Tissue} : \text{Infection} + \text{Tissue} : \text{keap1} + \text{Infection} : \text{keap1}$]. (A) Model-predicted vs actual expression of the nuclear factor (erythroid-derived 2)-like 2 (*nrf2*) gene in response to tissue variability, infection, and relative expression of the kelch-like ECH-associated protein-1 (*keap1*) gene, with tabulated analysis of variances (DF, F-value, and *p* value) obtained from the multiple linear regression. (B) Heatmap showing the parameter covariance matrix to illustrate the relationship between each parameter (listed on the right) in the model

Nrf2 orchestrates the intracellular redox state (DeNicola et al. 2011). In our current study, we observed basal expression of *nrf2* and *keap1* in various tissues, including adipose tissue, blood, brain, eye, fin, gills, heart, intestine, head kidney, liver, muscle, spleen,

stomach, and testes. Among these tissues, the liver, blood, and muscle exhibited high levels of *nrf2* expression, while *keap1* was prominently expressed in several organs (e.g., brain, liver, blood, spleen, eye, and head kidney). The liver and blood have direct interactions with stressors and are responsible for detoxification processes (Abdel-Mageid et al. 2020; Bawuro et al. 2018), hence the elevated expression of *nrf2* and *keap1* in these tissues, highlighting their important roles in antioxidant defense and cellular protection.

Interestingly, the head kidney and spleen displayed a similar pattern of *nrf2* expression. However, the spleen exhibited approximately seven-fold higher mRNA levels of *keap1* compared to the liver. This finding raises possible explanations, such as *keap1* having additional functions in the spleen or the head kidney being more susceptible to cellular damage, leading to lower *keap1* expression. Consequently, this reduced expression of *keap1* in the head kidney could result in the downregulation of *nrf2* expression during stress, allowing for rapid and extensive activation of antioxidant response element (ARE)-dependent antioxidant and detoxification defense mechanisms (Jiang et al. 2014; Nguyen et al. 2009). This notion is supported by evidence demonstrating that activation of Nrf2 through chemical inducers or genetic disruption of the *Keap1* gene significantly mitigates cellular damage caused by stress inducers (Ohkoshi et al. 2013; Suzuki and Yamamoto 2015).

It appears that tissues exposed to potential stressors, whether internal or external, tend to exhibit high expression levels of *nrf2* in order to maintain adequate levels of this protein. Among these tissues, muscles are particularly susceptible to oxidative stress and cellular damage due to factors like excessive exercise (Kitaoka 2021), which aligns with the observed high expression of *nrf2* in muscle tissue under homeostatic conditions. Furthermore, *nrf2* expression has been closely linked to mitochondrial function (Di Cristofano et al. 2021; Kitaoka 2021; Pan et al. 2022), suggesting that it plays a role in regulating energy metabolism. Studies have shown that the knockdown of *NRF2* leads to impaired mitochondrial function (Zweig et al. 2020).

Our findings indicate that under normal conditions, the transcript levels of *nrf2* in the stomach were higher than those in the intestine, although these increases were not statistically significant. Similarly, in the golden pompano (*Trachinotus ovatus*), *nrf2* transcripts were predominantly expressed in the liver and gut. However, the foregut and stomach exhibited relatively lower expression, which was still not significant compared to the midgut (Xie et al. 2021). These slight variations in gene expression among different species may be due to species differences or variations in experimental conditions. In contrast, when challenged with *A. hydrophila*, the expression of *nrf2* in the intestine was higher than in the stomach. Furthermore, in Chinese perch, intestinal *nrf2* expression, along with the associated antioxidant genes, was upregulated after periods of starvation (Pan et al. 2022). Similarly, in hybrid catfish and Jian carp, the expression of *nrf2* was also upregulated following dietary changes (Yu et al. 2022; Zhao et al. 2019). Taken together, the predominant expression of *nrf2* in the gut may be attributed to the high potential exposure of organisms to xenobiotics through the oral route, which subsequently requires elevated levels of *nrf2*.

Given the extensive research on the interplay between Nrf2, Keap1, and environmental stressors, our objective was to investigate how their expression changes in response to infection. Infections and diseases often lead to oxidative damage and apoptosis, while the Nrf2 pathway acts as a protective mechanism (Wang and Gallagher 2013) and plays a crucial role in regulating both innate and adaptive immune responses (Battino et al. 2018; Habte-Tsion 2020). In our study, we observed significant alterations in the expression of *nrf2* and *keap1* following the challenge with *A. hydrophila*.

Significant upregulation of *nrf2* was observed in key fish immune organs, including the liver, spleen, and head kidney, in response to inflammation. Conversely, *keap1* exhibited a

relatively low, but still upregulated, expression in these organs. The Nrf2/Keap1 signaling pathway activates the antioxidant response element (ARE), an enhancer sequence (a *cis*-regulatory element) located within the promoters of genes involved in detoxification and cytoprotection (Bhakkiyalakshmi et al. 2018). Notably, downregulation of Nrf2 in grass carp challenged with *A. hydrophila* resulted in decreased levels of detoxification enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase in the liver and blood (Ming et al. 2020). Infection with *A. hydrophila* has been shown to increase the activity of superoxide dismutase and catalase, as well as the levels of superoxide anion and H_2O_2 (Xia et al. 2017). These enzymes play a vital role in maintaining cellular redox balance by scavenging ROS (Suzuki and Yamamoto 2015). Thus, upregulating *nrf2* expression and maintaining low levels of *keap1* during inflammation are essential for suppressing oxidative stress.

It was evident that the first pathological sign observed after 1 h of infection with *A. hydrophila* was in the spleen of the channel catfish (Abdelhamed et al. 2017), which is consistent with the highest transcripts of *keap1* among the different tissues, along with a relatively low expression of *nrf2*. Furthermore, under normal conditions, there was a robust positive linear correlation between the expression of *nrf2* and *keap1*. However, during inflammation, this correlation weakened to a moderate level with a nonlinear relationship, indicating a disrupted Nrf2/Keap1 system in the spleen.

Interestingly, within 24 h of *A. hydrophila* infection, the kidney, liver, intestine, and gills exhibited pathological lesions (Abdelhamed et al. 2017). Notably, following the challenge with *A. hydrophila*, our findings revealed that these tissues demonstrated the highest levels of *nrf2* expression among all the tissues studied. Additionally, it is worth mentioning that the liver and head kidney are known to be target organs for the bacterium *A. hydrophila* (AlYahya et al. 2018). In these organs, we observed an upregulation of *nrf2* expression and moderate upregulation of *keap1* compared to other organs. It is important to note that the liver and kidney may later develop necrosis due to the cytotoxins produced by *A. hydrophila* (Lallier et al. 1984). On the other hand, other tissues, such as the skin, showed a lesser degree of affection, with the development of pathological lesions occurring later (AlYahya et al. 2018). Our findings indicated minimal changes in *nrf2* and *keap1* expression after infection with *A. hydrophila* in these tissues.

Of particular interest, the blood exhibited a robust linear correlation between the expression of *nrf2* and *keap1* under both normal and inflammatory conditions. This finding, along with previous studies, strongly suggests that the blood plays a pivotal role in mediating the Nrf2/Keap1 system in other tissues, such as the liver and gut. Furthermore, we observed strong correlations in the gills, intestine, and head kidney, but only moderate correlations in the liver and spleen following *A. hydrophila* challenge. However, in the normal condition, the correlations ranged from strong to fairly strong in these tissues, except for the intestine, which showed a moderate correlation. Given the intricate interplay between Nrf2 and Keap1, as evidenced by previous research (Bellezza et al. 2018; Hou et al. 2011), the variation in correlation strength across tissues raises intriguing questions. What factors contribute to the strong or moderate correlations in certain tissues and the weak correlation in others? Additionally, considering the ANCOVA results indicating a potential influence of bacterial infection on *nrf2* expression, could the type of infection (e.g., bacterial, viral, fungal, or parasitic) impact the reciprocal interaction between Nrf2 and Keap1? Could the embryonic origin of the tissue also play a role in mediating this interaction? Further investigation is warranted to uncover the underlying mechanisms governing the variations in correlation between the expression of *nrf2* and *keap1* across different tissues and determine whether this impacts the levels and activities of Nrf2 and Keap1. While Keap1 is known to

directly interact with Nrf2, it is possible that this interaction is influenced and assisted by other cellular molecules, such as heat shock protein 90 (Ngo et al. 2022). Notably, overexpressing Keap1 partially rescued the exacerbated toxic levels of Nrf2, whereas inhibition of heat shock protein 90 impaired this effect (Ngo et al. 2022).

While these findings enhance our understanding of *nrf2/keap1* crosstalk interactions in various tissues, it is important to note that they are based on gene expression analysis. Therefore, future investigations should focus on examining the protein expression of these molecules to obtain a more comprehensive understanding of this interaction.

Conclusion

In summary, this study reveals differential expression patterns of *nrf2* and *keap1* among various tissues. Under normal conditions, the liver and blood exhibit the highest expression levels of *nrf2* and *keap1*. However, upon infection, the liver, head kidney, gills, and intestine show increased *nrf2* expression, while the spleen displays high levels of *keap1*. This differential expression may contribute to the observed pathological features following infection, where signs of inflammation are detected earlier in the spleen and later in the liver, head kidney, gills, and intestine. Our correlation and expression analysis suggests that the blood plays a crucial role in mediating Nrf2/Keap1 signaling in other tissues. Furthermore, the spleen, gills, liver, head kidney, and intestine appear to be potential target organs of *A. hydrophila* based on these findings.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files. Further information related to the data involved in the manuscript can be obtained from Dr. Zizy I. Elbially (zeze_elsayed@fsh.kfs.edu.eg) or Dr. Abdullah Salah (abdallah_salah_2014@fsh.kfs.edu.eg) upon reasonable request.

Declarations

Ethics approval and consent to participate The study was conducted following international and national guidelines for animal studies, as well as the ethical guidelines for animal studies at Kafrelsheikh University, Egypt (approval number: IAACUC-KSU-2022–0021).

Consent for publication Not applicable.

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Authors and Affiliations

Zizy I. Elbially¹  · Abdullah S. Salah^{2,3}  · Ahmed Elsheshtawy^{1,3}  ·
Nasema M. Elkatatny⁴ · Alamira Marzouk Fouad⁵ · Haitham G. Abo-Al-Ela⁶ 

✉ Haitham G. Abo-Al-Ela
haitham.aboalela@frc.suezuni.edu.eg; haithamgamal2@gmail.com

¹ Department of Fish Processing and Biotechnology, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Kafrelsheikh 33516, Egypt

² Department of Aquaculture, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Kafrelsheikh 33516, Egypt

³ Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK

⁴ Biotechnology Department, Agricultural Research Center, Animal Health Research Institute, Dokki, Giza, Egypt

⁵ Aquatic Animal Medicine and Management, Faculty of Veterinary Medicine, Assiut University, Assiut 71526, Egypt

⁶ Genetics and Biotechnology, Department of Aquaculture, Faculty of Fish Resources, Suez University, Suez 43518, Egypt