# Identification and first characterization of 7 Septate Junction proteins (SJP) in decapod crabs and their potential involvement in osmoregulation

By

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## Abstract

Similar to tight junction proteins found in vertebrates, septate junction proteins (SJPs) serve a significant role in maintaining the epithelial barrier that regulates paracellular transport in invertebrates. Some limited knowledge exists of the cellular localization and function in insects, however, identification, tissue distribution and function of SJPs in crustaceans remains unknown. The current study investigated SJP homologs in three different decapod crabs: *Carcinus maenas*, *Metacarcinus magister*, and *Geothelphusa dehaani*. Seven SJP candidates - Megatrachea-like, Kune-kune-like, Contactin-like, Neurexin-IV-like, Würmchen1-like, Gliotactin-like, and Mesh-like- were identified and characterized using transcriptome mining and expression analysis based on sequence homology of known SJPs in insects. One candidate, Würmchen1-like significantly is shorter overall protein length (85 *vs*. 160 AAs) in compare to insect Würmchen1. A comparative mRNA expression analysis indicated that SJP expression patterns in the branchial epithelia are unique for each crab species, presumably due to a species-specific life style. Further, the study showed that SJPs are highly expressed in epithelial-rich tissues such as the gills and antennal glands of *C. maenas*, with much lower mRNA expression levels in muscle tissue, providing additional evidence that the investigated proteins are indeed SJPs. Relative mRNA expression of *megatrachea-like* and *kune-like* was highest among all SJPs in the anterior gills in *C. maenas*, regardless whether acclimated to seawater or brackish water. In marine *M. magister, würmchen1-like* was highest expressed, while *kune-like* showed highest mRNA expression levels in *G. dehaani*. When green crabs were acclimated to brackish water, branchial transcript abundance of all but *contacin-like* and *neurexin-IV*-like increased, suggesting roles for *megatrachea-like*, *kune-kune-like*, *würmchen1-like*, *gliotactin-like* and *mesh-like* in protecting for passive ion (e.g. $Na^+$, $Cl^-$, $Ca^{2+}$, and $K^+$) effluxes and/or water influxes. Exposure to high environmental ammonia, HEA (1 mM $NH_4Cl$), resulted in substantial but transient upregulation of all SJPs mRNA except *Mesh-like* in the posterior gills of hyper-regulating *C. maenas*. Interestingly, *Mesh-like* was the only SJP showing upregulation after a HEA exposure of 7 days.

Overall, it was demonstrated that the investigated transcripts most likely encode SJPs. However, further studies are necessary to verify their paracellular localization and barrier function.

## Acknowledgements

First and foremost, I would like to express my heartfelt gratitude to Dr. Weihrauch for all the time and patience over these past two years. I have learned so much from him, and he was always kind enough to answer my questions with great patience.

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Lastly, I am deeply grateful to my parents, who always encouraged me to keep learning and never remain idle. Their support and encouragement have always pushed me to take the next step forward.

## Thesis Contributions

The RNA samples of green shore crab tissues (gills, antennal glands, hindgut, claw muscles) were used from a previous study conducted by Mikyla Nash (Nash et al., 2022). The anterior gills of Japannes crab were provided by Dr. Wilson. Dr. Weihrauch assisted in editing the manuscript, study design, and provided financial assistance. All experiments and data were conducted, collected, analyzed, and presented by Mina Amiri Farahani.

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## List of Abbreviations

**ANOVA,** Analysis of Variance

**cDNA,** Complementary deoxyribonucleic acid

**CONT,** Contactin

**ECL,** ExtraCellular Loops

**Gli,** Gliotactin

**HEA,** High environmental ammonia

**JAM,** Junctional adhesion molecules

**Kune,** Kune-kune

***Mega,*** *Megatrachea*

**MAGUK,** Membrane-Associated Guanylate Kinase

***Nrx-IV,*** *Neurexin IV*

**ORF,** Open reading frame of gene

**PCR,** Polymerase chain reaction

**pSJs,** pleated Septate Junctions

**qPCR,** Quantitative polymerase chain reaction

**RNA,** Ribonucleic acid

**SEM,** Standard error of the mean

**SJP,** Septate Junction protein

***Sinu,*** *Sinuous*

**sSJs,** smooth Septate Junctions

**Ssk,** Snakeskin

**tTJ,** tricellular Tight Junction

**TJC,** Tight Junction Complex

**TJ,** Tight Junction

***Tsp2A***, *tetraspanin 2A*

***Wrm1***, *Würmchen1*

**ZO**, Zonula Occludens

# 1.0 Introduction

## 1.1 Tight Junctions Complex (TJC)

To maintain homeostasis, body fluids from any given animal must be separated from the environment. The most outer living cell layer is the epithelium, which is responsible for the regulated transport of small molecules, e.g. ions, water, monosaccharides, and amino acids, from the environment into the body fluids, and *vice-versa*. In general, an epithelium is a single layer of cells. The apical membrane of the cells is facing the environment, while the basolateral membrane is in contact with the body fluids. In order to guarantee a coordinated transepithelial transport, either uptake or excretion, the space between these cells, called the paracellular pathway, must be highly controlled. In vertebrates, this is accomplished by a tight junction complex (TJC), a complex composed of numerous proteins, of which some reach into the paracellular space, interacting with tight junction molecules of the adjacent cell. The TJC forms a sealing network, which restricts movements of larger molecules along the paracellular pathway, but only ions; these, however, only to a certain degree (Chasiotis et al., 2012a). Physiological and morphological studies have shown that the TJC forms a barrier in the paracellular pathway that is selective for ions with respect to charge and size according to the physiological requirements of the respective epithelium (Powell et al., 1981).

In general, there are two ways a TJC can be organized: (I) the bicellular tight junction (bTJ) is sealing the paracellular space between two adjacent cells. Here the main proteins are the occludins and claudins. (II) the tricellular tight junction (tTJ) where three cells connect together. The most important proteins that create such tricellular tight junctions are angulins and tricellulins (Günzel & Fromm, 2012 and Staehelin, 1973). The tricellular tight junction complex regulates the paracellular passage of macromolecules and water (Ayala-Torres et al., 2019).

Overall, the TJC is composed of a number of different proteins. In vertebrates, these include, among others, claudins, occludins, junctional adhesion molecules (JAM), and membrane-associated guanylate kinase (MAGUK) (Chasiotis et al., 2012b) (Fig. 1).

### *1.1.1 Claudins*

Claudins are tetraspan transmembrane proteins. Claudins have two extracellular loops (ECL) and four transmembrane domains. It is assumed that the extracellular loops determine the function of claudins, and it is hypothesized that the first larger extracellular loop is crucial to specifying the selective permeability of ions and the paracellular tightness. The second shorter extracellular loop is likely responsible for the narrowing of the paracellular cleft (Krause et al., 2008). Members of the claudin family are the most abundant transmembrane proteins of the TJC and are responsible for regulating the paracellular selectivity, forming paracellular pores for specific anions and cations (Günzel & Yu, 2013; Sasaki et al., 2003; Tsukita et al., 2019). The flexible structure of claudin strands is crucial in maintaining barrier function in cellular morphology and tissue regulation (Varadarajan et al., 2019). In vertebrates, numerous claudins exist. For instance, in a study on the pufferfish, *Takifugu rubripes*, 56 different claudins have been identified (Loh et al., 2004). This feature may be due to requirements found in fish, such as osmoregulation and adaptation in different salinity (Bagherie-Lachidan et al., 2008; Chasiotis et al., 2012 and Duffy et al., 2011).

### *1.1.2 Occludins*

Occludins were the first TJ proteins that have been discovered and identified in vertebrates (Furuse et al., 1993). An occludin is a tetraspan protein with three cytoplasmic domains, four transmembrane domains, and two extracellular loops that are tyrosine-rich. (Gonzalez-Mariscal et al., 2003). The role of occludins in the TJC is to maintain epithelial integrity. In addition, evidence suggests that occludins are the main factor in the regulation of barrier properties in epithelial and endothelial cells (Feldman et al., 2005).

### *1.1.3 Junctional Adhesion Molecules (JAMs)*

Junctional Adhesion Molecules are glycosylated transmembrane proteins that are localized at the TJC in endothelial and epithelial cells. JAMs belong to the immunoglobulin (Ig) superfamily and have three distinct parts: two extracellular Ig-like domains and a single transmembrane region.

The intracellular tail is short (Bazzoni, 2003; Ebnet et al., 2004). JAMs have been suggested to regulate the barrier function in epithelial and endothelial cells (Hartmann et al., 2020).

### *1.1.4 Membrane-Associated Guanylate Kinase (MAGUK) Proteins*

The MAGUK proteins belong to a protein family that include Zonula Occludens (ZO) proteins, e.g., ZO-1, ZO-2, and ZO-3. These proteins connect with TJ membrane proteins and the cytoskeleton. They also provide the structure for the assembly of the TJ complex within the cytoplasm. ZO-1 can be found in both in the TJC and in Adherence Junctions (AJs) (Bauer et al., 2010). ZO-1 is frequently described as a TJ "scaffolding" or "adaptor" protein. Therefore, it is assumed that ZO-1 has a double role: (1) to provide structural support to TJs by linking the actin cytoskeleton to claudins and occludins and (2) to convert signals to gene expression, cell differentiation, and proliferation through intracellular signaling pathways (Bauer et al., 2010).

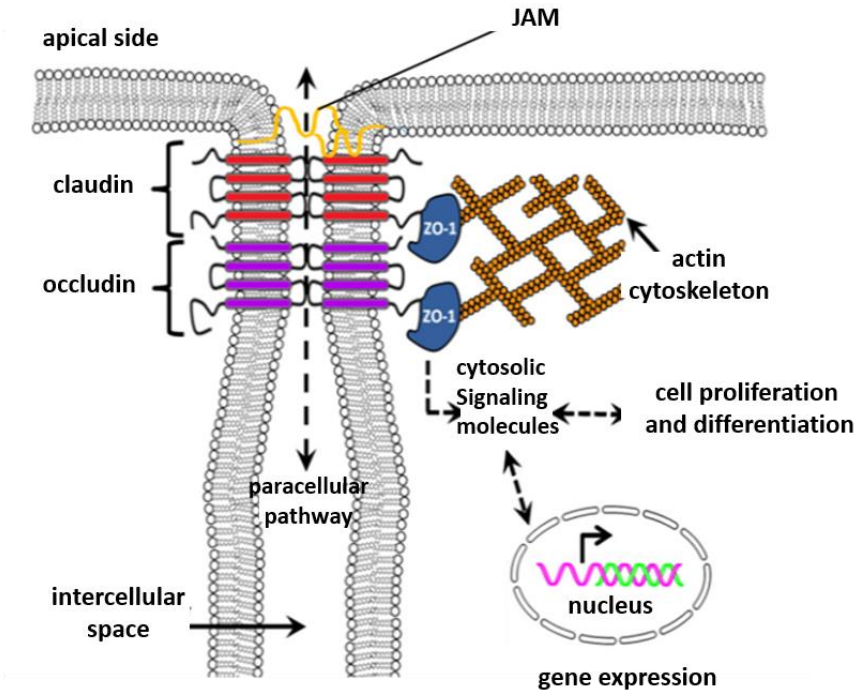


**Figure 1**: Localization of main proteins of the vertebrate Tight Junction Complex in the epithelia: claudins, occludins, JAM and ZO-1 in vertebrate epithelia (modified after Chasiotis et al., 2012).

## 1.2 Junction proteins in insects

The vast majority of studies on junction proteins have been conducted in insects, namely in the yellow fever mosquito, *Aedes aegypti*, and the fruit fly, *Drosophila melanogaster*. In invertebrates these junctions are called Septate Junctions (SJ) and they fulfil a similar function as their vertebrate counterparts, the tight junctions (Jonusaite et al., 2016a).

In *D. melanogaster* and likely other arthropods there are two main types of SJs: pleated Septate Junctions (pSJs) and smooth Septate Junctions (sSJs). Pleated SJs are generally found in tissues of ectodermal origin such as the epidermis, foregut, hindgut, trachea, imaginal discs, and salivary glands. Smooth SJs are observed in tissues of endodermal origin such as the midgut and the Malpighian tubules. However, currently there is little information about their function (Izumi & Furuse, 2014). Work from Beyenbach and others suggested that Mesh, Tetraspanin 2A, and Snakeskin are required for the assembly of the sSJs in the paracellular pathway in the apicolateral region and are important for the barrier function (Beyenbach et al., 2020). Besides characterizing the permeability of epithelia, it has been proposed that SJs might have a role in cell proliferation, cell communication, cell adhesion, and maintenance of cell polarity (Bilder et al., 2000; Bilder et al., 2003). Currently a number of membrane proteins have been investigated to be part of the Septate Junction Complex (SJC), including Megatrachea (Pickel), Kune-kune, Contactin, Neurexin IV, Würmchen, Gliotactin, and Mesh and less investigated Fasciclin III, Sinuous, and Tetraspanin 2A (Tsp2A).

### *1.2.1 The claudin-like family*

In insects, so far three claudin-like proteins, *Megatrachea* (Behr et al., 2003), *Kune-kune* (Nelson et al., 2010), and *Sinuous* (Wu et al., 2004), have been identified and investigated.

As shown in the vertebrate claudins, they feature 4 transmembrane domains and a conserved motif (W-GLW-C-C) localized in the first extracellular loop. Function of this motif in the claudins or claudin-like SJP has not been explored yet (Nelson et al., 2010; Wu et al., 2004, and Behr et al., 2003).

#### 1.2.1.1 Megatrachea (Mega)

The claudin-homolog, *Megatrachea (Mega)* or *Pickel*, was identified in *D. melanogaster* by Behr and others (2003). It has been shown to not only be important for transepithelial barrier function but also play a role in cell shape. In addition, *Mega* seems to be necessary for tracheal tube size control in *Drosophila* embryos (Behr et al., 2003). A Protter hydrophobicity analysis of the fruit fly *Mega* showed that the 256 AA molecule has four transmembrane domains with two extracellular loops, one intracellular loop, and the N- and a C-termini being located in the cytoplasm (Fig. 2). A sequence comparison between the insect *Mega* and the mouse claudin-18 (accession no. AF221068.1) and claudin-10 (accession no. AF124425.1) revealed a similarity of 37% and 41% and an identity of 19% and 20% in the amino acid sequence, respectively (Behr et al., 2003).

#### 1.2.1.2 Kune-Kune (Kune)

In insects *Kune* is expressed in the hindgut and likely plays an osmoregulatory role (Jonusaite et al., 2016b). Similar to Mega, the topology of *Kune* was predicted to be claudin-like. This 264 AA protein also consists of 4 transmembrane domains with two extracellular and one intracellular loop (Fig. 2). A knock-out of *Kune* in *D. melanogaster* embryos resulted in derangement and reduction abundance of the other SJs-like proteins including *Coracle*, *Megatrachea*, *Sinuous*, *Atpa*, *Discs large* (Dlg), and *Neurexin-IV*. Further, *Kune* is essential for the control of the tracheal tube size. (Nelson et al., 2010, and Van Itallie et al., 2006). A sequence comparison between the insect Kune and the mouse claudin-18 (accession no. AF221068.1) and claudin-10 (accession no. AF124425.1) revealed a similarity of 27% and 34% and an identity of 18% and 19% in the amino acid sequence, respectively.

#### 1.2.1.3 Sinuous (Sinu)

*Sinuous* (*Sinu*), in *D. melanogaster* a 247 AA protein, was first identified by Wu in 2004 (citation). As predicted for *Mega* and *Kune*, it is predicted to have four transmembrane domains, two extracellular loops, one intracellular loop, and an intracellular N and C terminus. *Sinu* is essential for SJC organization and has been shown to exhibit a paracellular barrier function (Fig.

2) (Wu et al., 2004). Similar to Kune, a sequence comparison between the insect Sinu and the mouse claudin-18 (accession no. AF221068.1) and claudin-10 (accession no. AF124425.1) revealed a similarity of 30% and 34% and an identity of 18% and 16% in the amino acid sequence, respectively.

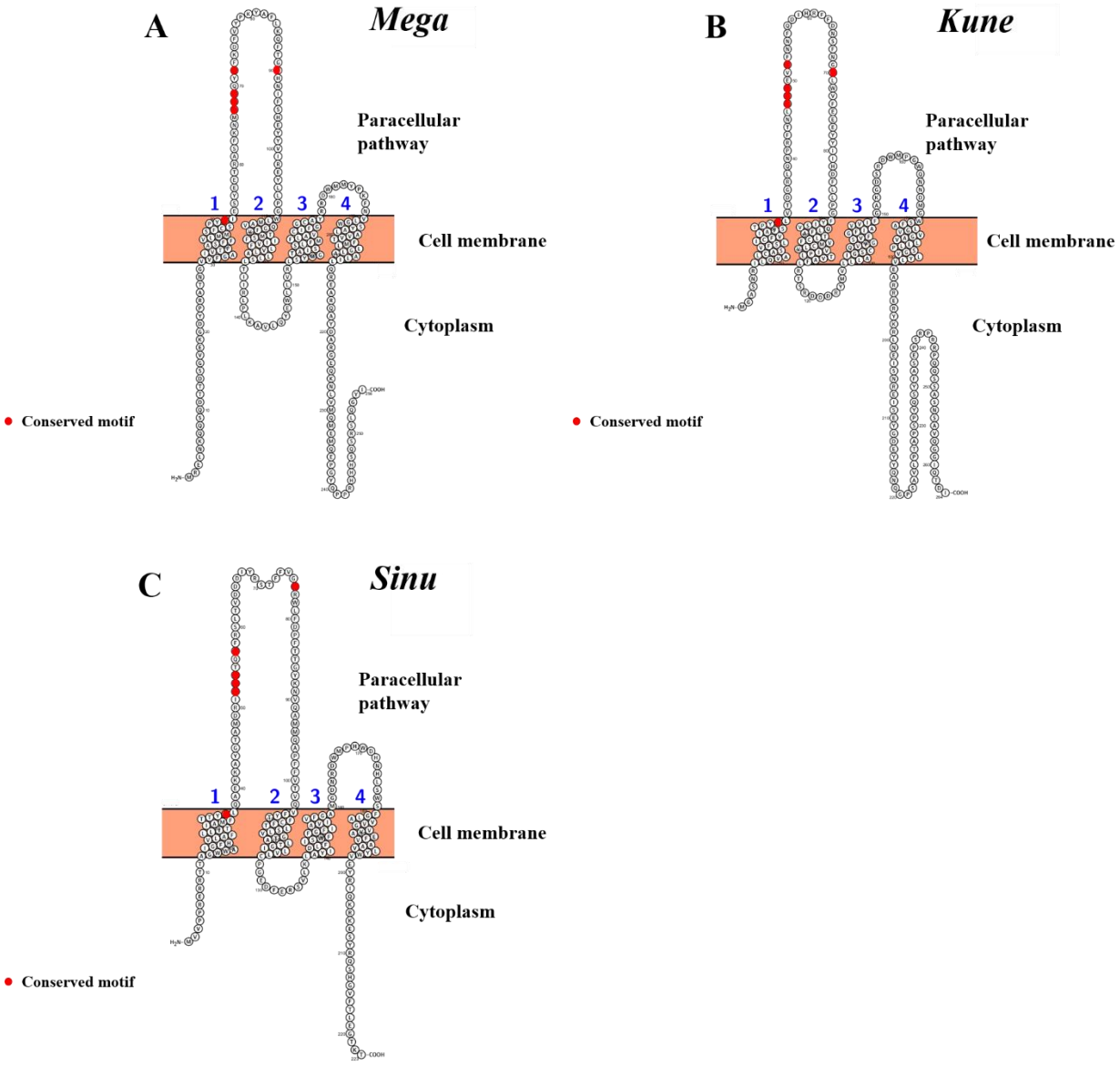


**Figure 2:** The predicted secondary structure of Claudin-like proteins (A) Mega (accession no. NP_569919.2), (B) Kune (accession no. NP_610179.2), and (C) Sinu (accession no. AAF50772.3) identified in *D. melanogaster*. Red dots indicate the conserved motif "W-GLW-C-C" found also in vertebrate claudins. The secondary structure was predicted using Protter (version 1.0, https://wlab.ethz.ch/protter/start/).

### *1.2.2 Contactin (Cont)*

In *D. melanogaster Contactin* is composed of 1390 AAs, and it is predicted to have one transmembrane domain, an extracellular region, and a short intracellular region (Fig. 3). A study in the fruit fly by Faivre-Sarrailh and other (2004) indicated that *Cont* plays a key role in SJ organization and is critical for paracellular barrier function (Faivre-Sarrailh et al., 2004).

It was further shown that *Cont* co-localizes with *Neurexin-IV* (see 1.3.3) and *Neuroglian*, an integral membrane glycoprotein (Bieber et al, .1989). Evidence was provided that the localization of *Cont*, *Neurexin-IV* and *Neuroglian* mutually dependent on each other at the SJC (Faivre-Sarrailh, 2004).

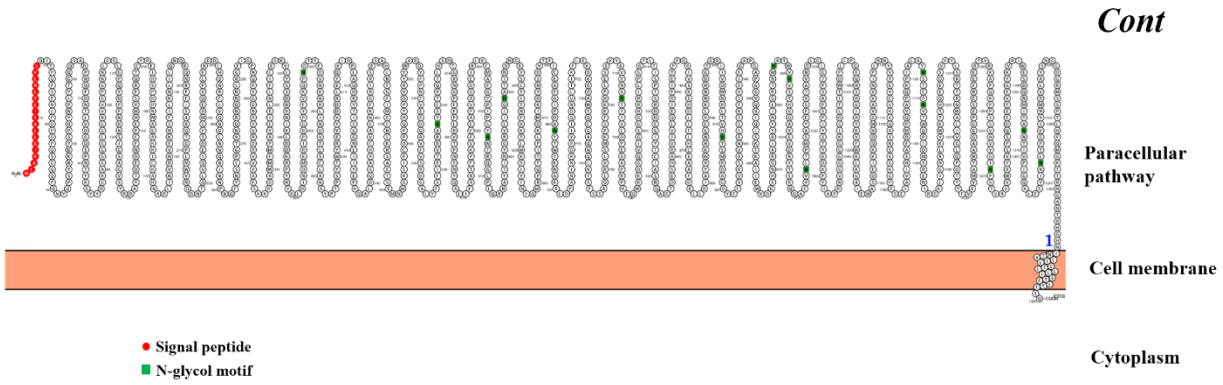


**Figure 3:** The predicted secondary structure of Contactin in *D. melanogaster* (accession no. NP_649461.2). Red dots indicate a signal peptide on the N-terminus; green dots indicate N-glycosylation motifs (Protter–version 1.0, https://wlab.ethz.ch/protter/start/).

### *1.2.3 Neurexin-IV (Nrx-IV)*

In *D. melanogaster Neurexin* is composed of 1284 AAs with one predicted transmembrane domain (Fig. 4). In the fruit fly it is mainly expressed in glial and epithelial cells. Glial cells have a variety of crucial functions, including the formation and establishment of blood-brain and blood-nerve barriers in the nervous system (Blauth et al., 2010). *Neurexin-IV* co-localizes with *Cont* and *Neuroglian* (Banerjee et al., 2006; Baumgartner et al., 1996; and Faivre-Sarrailh, 2004). Knock-down experiments in *D. melanogaster* suggested that *Nrx-IV* is necessary for proper epithelial tissue organization and survival (Ponte, 2019).

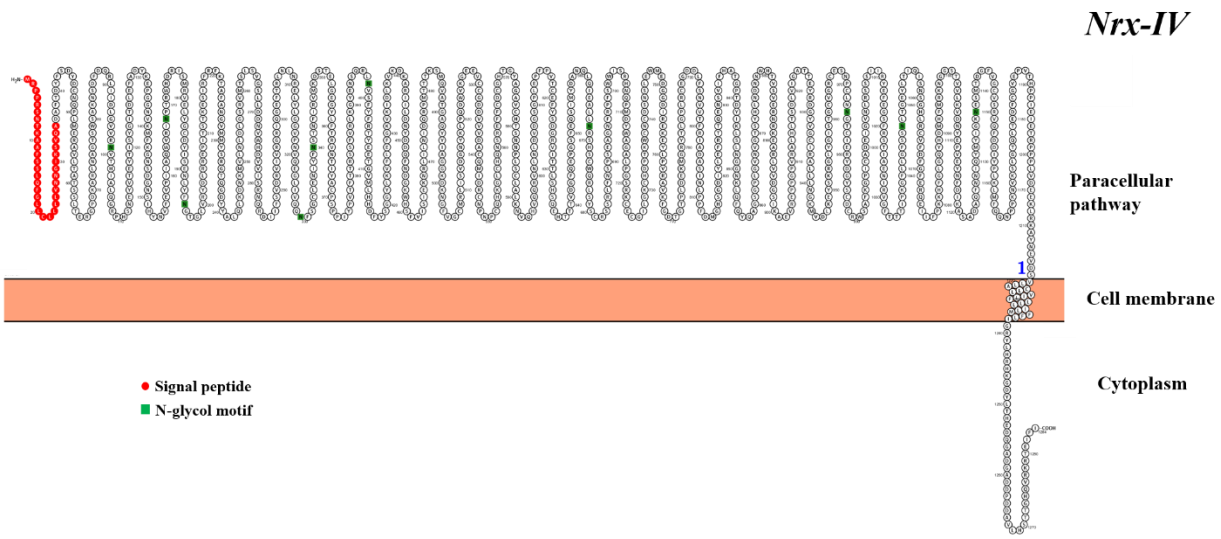


**Figure 4:** The predicted secondary structure of *Neurexin-IV* in *D. melanogaster* (accession no. NP_729787.2). Red dots indicate a signal peptide on the N-terminus; green dots indicate N-glycosylation motifs (Protter–version 1.0, https://wlab.ethz.ch/protter/start/).

### *1.2.4 Würmchen (Wrm)*

The short *Würmchen* (*Wrm*)1 & 2 proteins exhibit in insects one transmembrane domain and have been shown to be essential for normal epithelial tissue development (Fig. 5). While *Wrm2* is necessary for larval development, *Wrm1* (162 AAs) is required for the organization of the SJP-complex during embryogenesis. It is further essential for the barrier function of the paracellular pathway (Königsmann et al., 2020).

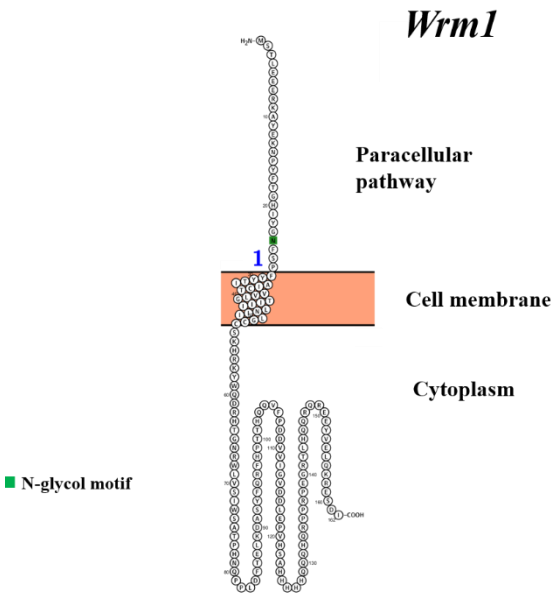


**Figure 5:** The predicted secondary structure of Würmchen 1 in *D. melanogaster* (accession no. NP_648105.1). Green dots indicate N-glycosylation motifs (Protter–version 1.0, https://wlab.ethz.ch/protter/start/).

### *1.2.5 Gliotactin (Gli)*

*Gliotactin* was the first SJ protein, that has been identified in *D. melanogaster* and is localized exclusively in the tricellular Septate Junction (tSJ), localized at regions between three adjacent epithelial cells. In *A. aegypti*, *Gliotactin* is composed of 993 AAs and contains one predicted transmembrane domain (Fig. 6). It is expressed in a diverse range of epithelial-like tissues including the epidermis, glial cells, trachea, hindgut, eye, and in the wings (Schulte et al., 2003). A knock-down of *Gli* in *A. aegypti* resulted in an increase of the epithelial permeability of the hindgut which was measured by the increase in the flux rate of polyethylene glycol (PEG-400) (Jonusaite et al., 2017a).

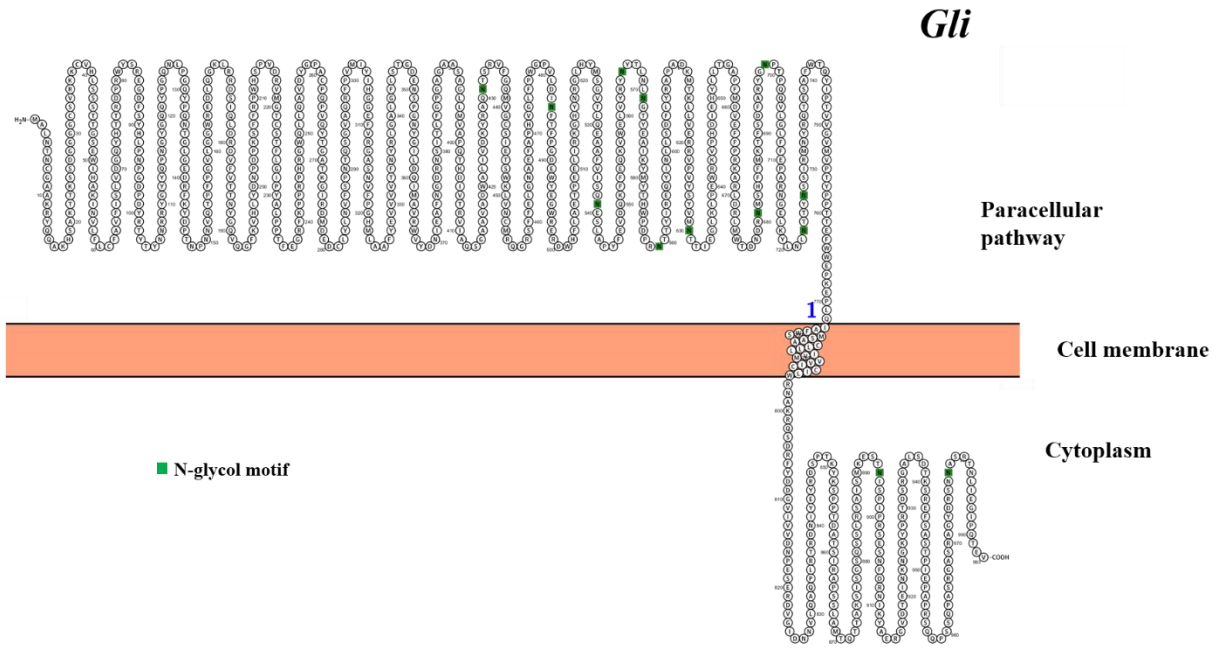


**Figure 6:** The predicted secondary structure of *Gliotactin* in *A. aegypti*. Green dots indicate N-glycosylation motifs (Protter–version 1.0, https://wlab.ethz.ch/protter/start/).

### *1.2.6 Mesh*

In insects *Mesh* is a smooth Septate Junction Protein (sSJP). In *D. melanogaster* it is composed of 1454 AAs and is predicted to have one transmembrane domain and a large extracellular region (Fig. 7). In *D. melanogaster*, *Mesh* is highly expressed and has been shown to be critical for the paracellular barrier function in the midgut (Izumi et al., 2012). In addition, *Mesh* is also required for physiological maturation of the Malpighian tubules and in this tissue it plays a crucial role in transepithelial ion transport (Jonusaite et al., 2023). In *Drosophila*, *Mesh* forms a complex with *Snakeskin* (Ssk), also a sSJP. Both proteins are interdependently related to each other in their localization and are essential for the organization and barrier function of the sSJC (Izumi et al., 2012).

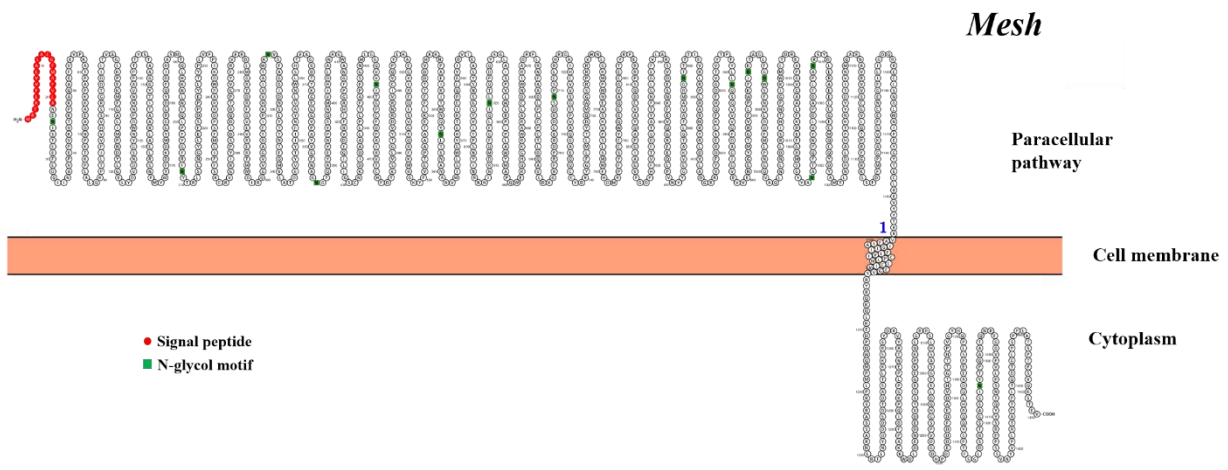


**Figure 7:** The predicted secondary structure of *Mesh* in *D. melanogaster* (accession no: NP_001163782.1). Red dots indicate a signal peptide on the N-terminus. Green dots indicate N-glycosylation motifs (Protter–version 1.0, https://wlab.ethz.ch/protter/start/).

### *1.2.7 Tetraspanin 2A(Tsp2A)*

*Tetraspanin 2A* (*Tsp2A*) is a smooth 244 AA Septate Junction Protein (accession no: XP_064556042), sSJP, that was investigated by Izumi in 2016 in *D. melanogaster* (Izumi et al., 2016). Here it was predicted that it has four transmembrane domains, two extracellular loops, and two intracellular regions (Fig. 8). The knockdown of *Tsp2A* in the Malpighian tubules of *D. melanogaster* was lethal in adult flies and it was concluded that *Tsp2A* is critical for the structure and function of the Malpighian tubules (Beyenbach et al., 2020).

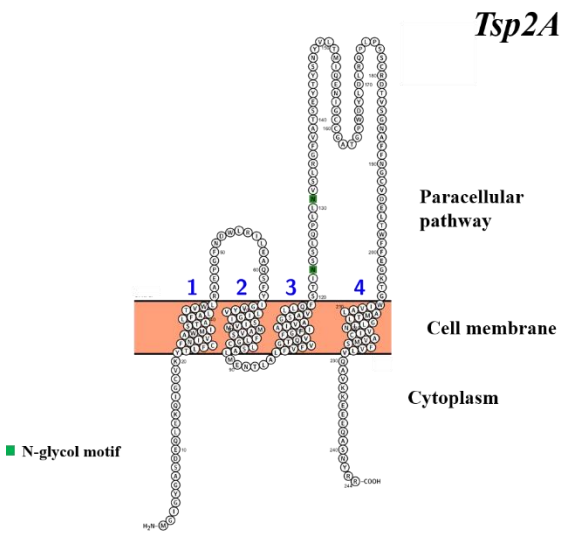


**Figure 8:** The predicted secondary structure of *Tsp2A* in *D. melanogaster* (accession no: AAF45608.1). Green dots indicate N-glycosylation motifs (Protter–version 1.0, https://wlab.ethz.ch/protter/start/).

### 1.3 Septate Junctions in crustaceans

In crustaceans little is known with regards to the Septate Junction Complex. To the authors' knowledge there are only two studies dealing with this subject. Employing transmission electron microscopy (TEM), Luquet and others showed that the SJC in the gill epithelium of the rainbow crab *Chasmagnathus granulatus* increased significantly in length from ca. 6 µm in animals acclimated to full strength seawater to ca. 11 µm when acclimated to a salinity of 12 ppt. (Luquet et al., 2002), implying a protective barrier function of this complex when animals hyperregulate in low salinity.

As part of a structural study on the gills of the green shore crab *Carcinus maeans*, Goodman and Cavey published a TEM image that displays the SJC in the tissue (Fig. 9) (Goodman & Cavey, 1990).

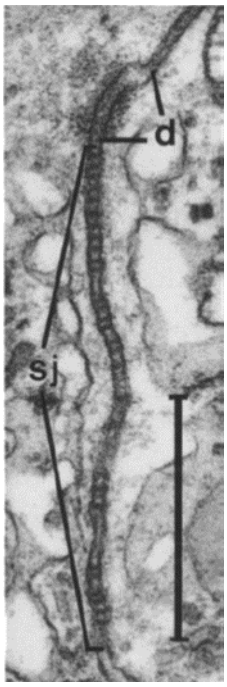


**Figure 9**: Transmission electron micrograph of junctional complex between branchial epithelial cells in *C. maenas*. Sj, Septate Junction, d, desmosome. Magnification: 72200 x; Scale bar: 0.5 µm. (modified after Goodman & Cavey, 1990).

As mentioned above, the SJC in crustaceans responded to changing environmental salinity. Therefore, we choose to investigate 3 different decapod crabs: The fully marine Dungeness crab, *Metacarcinus magister*; the euryhaline green shore crab, *Carcinus maenas*, and the Japanese freshwater crab, *Geothelphusa dehaani*.

### *1.3.1 Experimental animals*

#### 1.3.1.1 The green shore crab *Carcinus maenas*

The green shore crab, *Carcinus maenas*, is a euryhaline decapod crab native to the Northeast Atlantic Ocean and the Baltic Sea. The crabs mature within 1-2 years with a total lifespan of 3-5 years. Adults can reach a carapace width of 6-9 cm, though invasive populations can vary in size due to environmental factors such as salinity, temperature, and food availability. (Grosholz & Ruiz, 1996; Behrens & Hunt, 2000). Due to its physiological robustness, it has become one of the most widespread invasive species across North America, South Africa, Australia, and parts of Asia (Frederich & Lancaster, 2023; Behrens & Hunt., 2000; Carlton & Cohen, 2003). *Carcinus maenas* typically inhabits intertidal and shallow subtidal zones, ranging around 0 to 10 meters in depth. It

can often be found in estuaries, rocky or muddy shores, and salt marshes. The crab can survive salinities of 4-34 ppt and even up to 52 ppt (Cohen & Carlton, 1995). When at rest, the green crab burrows into the soft sediments. During low tide it can also be found scavenging on the shore (hence its name shore crab), but often hiding under rocks and algae to avoid evaporation. This euryhaline crab is a hyperregulator when in dilute media, promoting an active NaCl uptake over its branchial epithelium (Henry & Weihrauch, 2023; Péqueux et al., 1988).

#### 1.3.1.2 The Dungeness crab *Metacarcinus magister*

The Dungeness crab, *Metacarcinus magister*, is a stenohaline, osmoconforming crab that inhabits full strength seawater. Dungeness crabs can be found in different substrates, including, mud, sand, rock, and graval (McGaw, 2005; Carroll & Winn, 1989). The crabs fully mature within 4 years with a total lifespan of 8-10 years (Ricketts et al., 1985). The Dungeness crabs bury in the substrate during periods of inactivity (McGaw., 2005; Carroll & Winn, 1989). The size of crabs in commercial and sport fisheries varies from 14.5 to 16.5 cm (Rasmuson, 2013).

#### 1.3.1.3 The Japanese freshwater crab *Geothelphusa dehaani*

The Japanese crab, *Geothelphusa dehaani*, belongs to Potamidae, which is a family of freshwater crabs. *G. dehaani* is a species endemic to Japan. Here it inhabits rivers, swamps, and streams (Suzuki, 2012). The crabs mature in the 3$^{rd}$ year with an estimated life span of 10 years. Adults reach a carapace width of 2-3 cm **(**Araki & Matsuura, 1995).

## 1.4 Aims and Objectives

The objectives of this study are:

1) Identification of major SJC proteins at the mRNA level in decapod crabs.
2) Evaluation of *transcript abundance* of SJPs in gills of crabs restricted to full strength seawater (Dungeness crab, *Metacarcinus magister*), freshwater (Japanese crab, *Geothelphusa dehaani*), and in the euryhaline green shore crab *Carcinus maenas* acclimated to either brackish water or seawater.

3) Determination of mRNA expression levels in various tissues in the hyper-regulating green shore crab, *C. maenas.*
4) Evaluating the effect on mRNA expression levels of SJPs in the NaCl transporting posterior gills and the antennal gland of *C. maenas.*
5) Determination of the effect of exposure to elevated environmental ammonia concentrations on the mRNA expression levels in the posterior gills of brackish water acclimated *C. maenas*.

## 1.5 Hypotheses

1) Since crustaceans belong to the same phylum as insects (Arthropoda), it is predicted that SJ-like proteins identified in insects will also be found in crabs.
2) It is hypothesized that the relative mRNA expression pattern in the anterior gills are similar in crabs inhabiting the same environment, such as the SW dwelling Dungeness crab and the SW acclimated green shore crab. It is further expected that the mRNA expression pattern of the SJPs is different in gills from animals acclimated to different salinities as the ionic gradients, then therefore also the requirements for the paracellular "tightness", are different.
3) Among the anterior and posterior gills, antennal gland, hindgut, and claw muscle, in the osmoregulating green crab, transcript abundance of SJ proteins is predicted to be highest in the gills, as this epithelium is directly exposed to the environment, which has a considerably lower osmolality when compared to the hemolymph. Between anterior and posterior gills, it is expected that the NaCl-transporting posterior gills will exhibit even higher expression levels than the anterior gills to avoid unwanted re-fluxes of ions *via* the paracellular pathway. Lowest expression is expected in claw muscle tissues, as this contains no epithelia, but SJP are assumed to be expressed in epithelia only.
4) It is hypothesized that the osmoregulatory organs, namely the posterior gill and the antennal gland, respond to salinity change. Thus, it is assumed that the mRNA expression levels of SJPs involved in the barrier function for salinity-related ions increase in brackish water acclimated crabs.
5) It is hypothesized that exposure to elevated environmental ammonia levels causes an upregulation of mRNA expression of SJPs related to paracellular $NH_4^+$ fluxes in order to protect from unwanted influxes of toxic ammonia.

# 2.0 Methods

## 2.1 Animals

### *2.1.1 Carcinus maenas*

Some of the total RNA used for the described experiments on *C. maenas* was available from previous experiments conducting similar research (Nash et al., 2022). Animals for all conducted experiments were male crabs caught from Northern Placentia Bay, NL, Canada and then transferred to the Animal Holding Facility at the University of Manitoba (Winnipeg, MB, Canada). All green crabs were kept for at least 2 weeks in either 1200-gallons of recirculating artificial seawater (32 ppt., pH 8.1, 14 ºC; Fritz Reef Pro Mix (RPM), Fritz Aquatics, Mesquite, TX, USA) or recirculating 300-gallon tanks of brackish water (10 ppt., pH 8.1, 14 ºC, Fritz RPM). The light: dark cycle was kept to 14:10 hours for both conditions. Crabs were provided plastic shelters and fed frozen scallops *ad libitum* 3-times per week.

### *2.1.2 Metacarcinus magister*

Male Dungeness crabs were purchased from a local supermarket (The Real Canadian Superstore, Winnipeg, Manitoba, Canada) and housed in recirculating seawater systems (1200-gallons tanks) in the Duff Roblin Animal Holding Facility at the University of Manitoba for a minimum of 7 days prior to experimentation. The artificial seawater (Fritz RPM, U.S.A.) was adjusted to a salinity of ca. 32 ppt, pH 8.1, and a temperature of 14 °C. The light regime was set on a 14:10 hour light: dark cycle. Crabs were provided plastic shelters and fed frozen scallops *ad libitum* 3-times per week.

### *2.1.3 Geothelphusa dehaani*

Male *Geothelphusa dehaani* crabs were obtained from a fish market in Vancouver (Canada) and transferred to the animal holding facility at Wilfrid Laurier University (Canada). Upon arrival animals were kept in artificial freshwater (499 µM $K^+$, 40 µM $Ca^{2+}$, 199 µM $Mg^{2+}$, 1 µM $Mn^{2+}$, 500 µM $Na^+$, 500 µM $HCO_3^-$, 600 µM $Cl^-$, pH = 7.6-7.8). Anterior gills were dissected under RNase free conditions, placed in RNA-later and then shipped to Winnipeg for further processing.

All experimental crabs, *C. maenas*, *M. magister*, and *G. dehaani*, were fasted for 48-hours prior to experimentation to mitigate the effects of feeding on the animal's physiological state.

### 2.2 Exposure to high environmental ammonia (HEA)

For HEA exposure experiments brackish water (10 ppt salinity) acclimated male *C. maenas* crabs were transferred to smaller 20-liter tanks- also aerated brackish water- and fasted for 2 days. Then the tank water was replaced with brackish water (10 ppt, pH 8.1, 14ºC, Fritz RPM) which was enriched with 1 mM $NH_4Cl$. The tank water was replaced daily. Animals were sampled after 2 days of exposure or, in a parallel set-up, after 7 days of exposure. No mortality was observed during HEA exposure.

### 2.3 Identification of SJP in crustaceans

In order to mine the transcriptomes of *C. maeans* and *M. magister* a tblastn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was conducted employing published insect protein sequences. Megatrachea (a.k.a. Pickel) (accession no. NP_569919.2), Kune-Kune (accession no. JV217000), Contactin (accession no. NP_649461.2), Neurexin-IV (accession no. CAA60383.1), Würmchen 1 (accession no. NP_648105.1), and Mesh (accession no. NP_001163782.1) protein sequences from *Drosophila melanogaster* were utilized for the search. For the transcriptome search of Gliotactin, the protein sequence from *Aedes aegypti* (accession no. KX823345.1) was used. For all obtained crab sequences (*C. maeans* and *M. magister*) gene-specific primers were designed (see table 1) and tested on cDNA synthesized from DNased (DNase I, Invitrogen, MA, USA) total RNA (0.5 µg) isolated from branchial tissues (see below) by endpoint PCR. The obtained PCR products were gel purified (E.Z.N.A Gel Extraction Kit, Bio Tek, Winooski, VT, USA) and sequenced (Centre for Applied Genomics, Toronto, ON, CA) for verification.

Currently no transcriptome information exists for the freshwater crab *Geothelphusa dehaani*. In order to obtain sequence information for all 7 SJPs in this freshwater crab, mRNA sequences for individual SJPs from *C. maenas*, *M. magister*, and the Chinese mitten crab, *Eriocheir sinensis*, were aligned to find highly conserved regions. Degenerate primers were then

designed (primers labeled with “crab”) targeting those regions to obtain an initial sequence for an individual SJP by endpoint PCR (annealing temperature: see table 2) in *Geothelphusa dehaani*. As before, initial PCR products were gel-purified and verified by sequencing (see Appendix 1). The transcriptome of *M. magister* did not contain the sequence for Mesh. Accordingly, degenerate primers were designed based on conserved regions of Mesh found in *C. maenas* and *Eriocheir sinensis*ccession no. XP_050734890.1) (see table 3).

## 2.4 Quantitative PCR

### *2.3.1 Relative mRNA expression of SJPs*

After a 30 min chilling period on ice, anterior gills (gill pair 5) from seawater (32 ppt salinity) and brackish water (10 ppt salinity) acclimated *C. maenas*, marine *M magister* (32 ppt salinity), and freshwater *G. dehaani* were dissected under RNase-free conditions and stored in RNAlater (Applied Biosystems, Austin, TX, USA) at -80ºC until RNA isolation. Total RNA was isolated from tissues with TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA). The quality and quantity of the RNA was monitored spectrophotometrically using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Ottawa, ON, CA). Moreover, the integrity of RNA was checked by gel electrophoresis on a 1% agarose gel containing ethidium bromide for visualization. Prior to the synthesis of cDNA, 0.12 μg total RNA was employed for experiments comparing relative SJP expression strength (Fig. 25) and 0.5 μg total RNA for all remaining experiments. RNA was treated with DNase I (Invitrogen, MA, USA) to remove any potential DNA contamination. Success of the DNase treatment was verified by the failure to amplify transcripts encoding the ribosomal Protein S3 (RbS3) (see table 1) *via* PCR. Complementary DNA (cDNA) was then synthesized from 0.12 or 0.5 μg DNase-I-treated RNA using the LunaScript® RT SuperMix Kit (New England Biolabs, Ontario, Canada). Successful cDNA synthesis was evaluated by the amplification of RbS3 *via* PCR. All PCR products were assessed by ethidium bromide-stained TAE agarose gel electrophoresis.

For quantitative PCR, species-specific and gene-specific primers were used (see table 1) after testing for optimal annealing and efficiency conditions by running a serial dilution of the respective cDNA. For qPCR, the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used. A melt curve analysis was performed once the cycling protocol was complete to verify the

presence of a single amplified product. A minimum $R^2$ value of 0.9 was required for all standard curves used with a PCR efficiency of ≥ 90%.

Due to the varying experimental conditions (salinity, HEA) and/or the use of various tissue, finding a non-changing internal reference gene was difficult. The ribosomal protein S3 was used as the reference gene to determine the relative expression strength of the SJP in *C. maenas* (see table 1) and for *M. magister* the $Na^+/K^+$-ATPase (α-subunit) was used (see figure 25). PCR products were verified by sequencing (Centre for Applied Genomics, Toronto, ON, CA). For all other qPCR experiments no non-changing internal standard with sufficient efficiency were found. In these cases, the NORMA-gene method (Heckmann et al., 2011) was applied, allowing a relative quantification without an internal standard gene.

**Table 1:** Target genes and primer sequences employed in qPCR for septate junction protein.

| Transcript | GeneBank accession no. | Primer (5'→ 3') | Product size (bp) | Annealing temp. (◦C) |
|---|---|---|---|---|
| ***Megatrachea (C. maenas)*** | GFXF01000913.1 | Sense: GTTCCATGGTCAATG Antisense: CAGAACTCCCACAAGCCCAT | 136 | 60 |
| ***Neurexin IV (C. maenas)*** | GFXF01000027.1 | Sense: TTCTACAATGACGGCCAGGT Antisense: TCCATGCCAGCGTCATAGAT | 122 | 60 |
| ***Mesh (C. maenas)*** | GBXE01116262.1 | Sense: CGATGAGGCTAACAAGGTGC Antisense: CTGGTACCCGACACAGTCTT | 151 | 60 |
| ***Kune-kune* (C. maenas)*** | GFYV01332919.1 | Sense: TCCAGCATTCTTCTTCGTGACAG Antisense: CGATGAAGCCAAAGAACCAT | 173 | 53 |
| ***Kune-kune (C. maenas)*** | GFYV01332919.1 | Sense: AATCGTGACTGGATGCCACA Antisense: GGATGCGATACTCCACCCAG | 121 | 59 |
| ***Contactin (C. maenas)*** | GFYW01020897.1 | Sense: CCCACAGAAGTGTCTGCCTT Antisense: TTTGCCTCGGATGTTCTCCC | 93 | 60 |

| | | | | |
|---|---|---|---|---|
| ***Würmchen1 (C. maenas)*** | GFYV01022712.1 | Sense: AGCCTTCAACAACACCTTGC Antisense: GACGATGGCCAAGATAGTGC | 115 | 58.6 |
| ***Gliotactin (C. maenas)*** | GBXE01097000.1 | Sense: AGATGCCCCTCCACTTGTAC Antisense: CCAGCCGGTTGTCATTGAAC | 111 | 60 |
| ***$Na^+/K^+$-ATPase*** | KJ133272.1 | CancerNaKF1: CAACCGTGCTGAGTTCAAGA CancerNaKR2: TCCACACACTTCAGCAAAGC | 118 | 60 |
| ***Megatrachea (M. magister)*** | GHOI01010124.1 | Sense: GGTATCGTGGCCTTTGTTGC Antisense: AAGCAGAACTCCCACAGTCC | 122 | 58 |
| ***Neurexin IV (M. magister)*** | GHOI01007751.1 | Sense: GACAATTACGAGCCGGTGTC Antisense: TGTCATCAAACTCCACCCGA | 148 | 60 |
| ***Kune-kune (M. magister)*** | GHOI01014120.1 | Sense: AGCCCCATTCTTTGTGACGG Antisense: TTGAGGACCGAGCGTTCAAA | 126 | 54 |
| ***Würmchen1 (M. magister)*** | GHOI01037666.1 | Sense: TCATCATCTGCACCCTCCTT Antisense: ATGAGGGAGGCAAATCGGTT | 121 | 62 |
| ***Gliotactin (M. magister)*** | GHOI01016740.1 | Sense: AAAGGACTGGCACGTTGTTC Antisense: AGCCGGTTGTCATTGAACAC | 132 | 59 |
| **RbS3F1R1 (crab)** | JF276909.1 | Sense: GTCCCTTTTCACCAAGGACA Antisense: CAAGGCCAAACTCAACAGGTT | 160 | 60 |
| ***Megatrachea (G. dehaani)*** | Unpubl. | Sense: GCTGTGGGAGTTTTGCTTCA Antisense: GTCTGCACTGCCATAAACCA | 144 | 60 |

| | | | | |
|---|---|---|---|---|
| ***Neurexin IV (G. dehaani)*** | Unpubl. | Sense: CGTCTACATCGGCAAGAACG Antisense: GAGGACGTTGACTCTGCTGT | 141 | 57.5 |
| ***Kune-kune (G. dehaani)*** | Unpubl. | Sense: GTTCTTCGGGTTCATCGCAG Antisense: CCACAACTCCTACCACACCT | 122 | 60 |
| ***Contactin (G. dehaani)*** | Unpubl. | Sense: CAATGCCACCTCCCTCAATG Antisense: CTGTCTCACTGTCCTTCCGA | 116 | 57.8 |
| ***Würmchen1 (G. dehaani)*** | Unpubl. | Sense: TGGAATCAAGCCAGAATTCTACA Antisense: CTGCAGATCCAGTTGACCAT | 102 | 59 |
| ***Gliotactin (G. dehaani)*** | Unpubl. | Sense: ACATCCGCTTCTTCAATGGC Antisense: ATAATCCTTGGTCCGTGGCA | 107 | 60 |

*Utilized only in tissue comparison

**Table 2:** Primer sequences employed in *Geothelphusa dehaani* for sequencing of septate junction protein. *C.m*., *Carcinu maenas*; *M.m*., *Metacarcinus magister*. Y=C+T; R=A+G; K=G+T; B=C+T+G; N=A+C+T+G

| Transcript | GeneBank accession no. | Primer (5'→ 3') | Product size (bp) | Annealing temp. (◦C) |
|---|---|---|---|---|
| ***Megatrachea* F1R1 (crab)** | Consensus seq. From *C. m*. and *M. m*. | Sense: GCTGTGGGAGTTTTGCTTCA Antisense: AGCCAATCTCGAGACCAACA | 345 | 50 |
| ***Neurexin IV* F1R1 (crab)** | Consensus seq. From *C. m*. and *M. m*. | Sense: TCCAGTACGTCTACATCGGC Antisense: TCGATCCCGAGTAGTCTTCGA | 173 | 50 |
| ***Gliotactin* F3R3 (crab)** | Consensus seq. From *C. m*. and *M. m*. | Sense: GCGBYACRTYATGAACGTGTC Antisense: TTRCGCACSAGCCGGTTGTC | 996 | 60 |
| ***Kune-kune* F3R4 (crab)** | Consensus seq. From *C. m.* and *M. m*. | Sense: TCGCCTACATCGACCTYTTC Antisense: GTYCCCTCNARGGTRAAGACKCC | 249 | 60 |
| ***Würmchen1* F5R6 (crab)** | Consensus seq. From *C. m*. and *M. m*. | Sense: TGCCTGAGTTCAACAATCRGT Antisense: GTACTYKGAGTGTTCGTGGCAG | 149 | 59 |
| ***Contactin* F1R2 (crab)** | Consensus seq. From *C. m*. and *M. m*. | Sense: CGCACCAAGTACTTCTACAC Antisense: CGTTCCAAGAACCGCTCACTCTC | 401 | 53 |

**Table 3:** Primer sequences employed in *Metacarcinus magister* for sequencing of mesh-like.

| Transcript | GeneBank accession no. | Primer (5'→ 3') | Product size (bp) | Annealing temp. (◦C) |
|---|---|---|---|---|
| ***Mesh* F6R6 (crab)** | Consensus seq. *C. m.* and *Eriocheir sinensis* (XP_050734890.1) | Sense: CGGNACCAGAATCTACCTCC Antisense: GATGTTGACGAACTGGTCCT | 447 | 51 |

### 2.5 Relative transcript abundance of SJ- like protein in gills of *Litopenaeus vannamei*

We used the data available from the Crustybase website (https://crustybase.org/) and a tblastn search function on the website to obtain transcript abundance of SJ- like protein, *mega-like*, *kune-like*, *contactin-like*, *neurexin-IV*, *wrm1-like*, *gliotactin*, and *mesh-like*. The transcriptome project was conducted on the gills of whiteleg shrimp, *Litopenaeus vannamei*, which is a decapod crustacean that was exosed for 30 days to either seawater (salinity = 30 ppt), brackish water (salinity = 15 ppt), or freshwater (N=2) (Zhang et al., 2016). In their expression analysis the authors have not considered potential SJPs.

### 2.6 Statistical analysis

Relative mRNA expression of SJPs in anterior gills was assessed using a one-way ANOVA with Game-Howell post hoc pairwise comparisons and Student's t-test in *C. maenas* (SW and BW). In *G. dehanni*. however, relative mRNA expression of SJPs in anterior gills was assessed using only a one-way ANOVA with Game-Howell post hoc pairwise comparisons (Fig. 25). The mRNA abundance of septate junction proteins in selected tissue was assessed with a one-way ANOVA with Game-Howell post hoc pairwise comparisons and Student's t-test in *C. maenas* (BW) (Fig. 26). The effect of salinity on mRNA expression in osmoregulating posterior gills and the antennal gland of seawater and brackish water acclimated *C. maenas* was assessed with a Student's t-test (Fig. 27).

The effect of HEA on mRNA expression in posterior gills was assessed with a Student's t-test in *C. maenas* (BW) with both the 2 day exposure and the 7 day exposure was tested individually against the control values (Fig. 28).

All data sets (p values < 0.05) were considered significant. The p values between 0.1 and 0.05 were indicated in the respected graphs. Data are presented by graphs as means ± standard error (SEM).

# 3.0 Results

## 3.1 Identification of Septate Junction proteins in *Carcinus maenas, Metacarcinus magister,* and *Geothelphusa dehaani*

Since Septate Junction proteins (SJPs) have only been characterized in insects, the initial goal of this study was to identify insect-like SJPs also in decapod crustaceans, which are grouped together with insects in the clade of Pancrustacea (Weihrauch and O'Donnell, 2015).

### *3.1.1 Megatrachea (Mega)*

Transcriptome mining employing the published amino acid sequence for Mega in *Drosophila melanogaster* (accession no. NP_569919.2, also called Pickel) allowed to identify Mega-like proteins from *C. maenas* (accession no. GFXF01000913; 258 AA; 37% identity and 58% similarity in the AA sequence to *D. melanogaster*) and *M. magister* (accession no. GHOI01010124.1). Nucleotide sequences from both crabs were then used to design crab-specific primers to obtain a 335 bp transcript coding for a partial Mega-like protein (111 AA) expressed in the gills of the freshwater crab *Geothelphusa dehaani* (see Appendix I). As seen in Fig. 10, the deduced amino acid sequences from the crab Mega-like protein share a high degree of identities among each other. Applying a tblastn search revealed between the obtained full open reading frame (ORF) of the Mega-like protein from *C. maenas* and other true crabs, including *Pachygrapsus marmoratus* (accession no. GIJU01018998.1), *Scylla olivacea* (accession no. GDRN01078484.1), *Eriocheir sinensis* (accession no. GFBL01086149.1), and *Hyas araneus* (accession no. HACH01025925.1) a ≥ 81% identity in the AA sequences. It must be considered, however, that published crustacean transcriptome sequences have not been verified for correctness.

```
C. maenas.        MDDKRSWRSSVGILQNGQSWDSMHRDIKPTPPSSSMVNAGIVAFVGTIFFIISFVSPYWL        60
M. magister       MDDKRSWRSSVGILQNGQSWDSIGRDIKPSPPSAALVNAGIVAFVATVFFVISFTSPYWL        60
                  ********************  ***** ***   ********* * ** *** *****

C. maenas.        QSYSYTYSDFKNMGLWEFCFDGFRYPKYQYDQKFMGCNYIFSEEYRIIWQWMIPAWFMSV        120
M. magister       QSYSYTYSDFSNMGLWEFCFDGFRYPKYQYDHKFLGCNYIFSEEYRIIWSWMLPAWFMAV        120
G. dehaani.       --------------LWEFCFNGFRYPKYQYDYKFMGCNYIFSEEYRIIWSWMIPAWFMAV        46
                  ********** *********:********** **:**************.**:*****:*

C. maenas.        QTFMTIALLGTMTTLVTVSLILTRWPLQMVMRYEWHLTGFCFLCQALSVVAIFFAVLVFG        180
M. magister       QTFMTIALLASMTTLVTVSLILMRWPMQMIMRYEWHLTGFCFLCQAVTVVALFFAVLVFG        180
G. dehaani        QTFMTIALLASMTTLVTVSLILVRWPMQMIMRYEWHLTGFCFLCQAVTVVAIFFAVLVFG        106
                  *********.:*********** ***:**:****************::***:********

C. maenas         VMCWSRDWLLNPNFNYLSWSYAFAVVAGGFHAFSGFVLLYETFEARERKRQATSMLHMDP        240
M. magister       VMCWSRDWLLNPNFNYLSWSYAFAVVAGGFHAFSGFVLLHETFEARERKRQATNLLHMDQ        240
G. dehaani        VMCWS-------------------------------------------------------        111
                  ************************************ ************  *****

C. maenas        QGQMGMGMGMGMVSQGYI   258
M. magister      QGHMGMGMGMGMVSHGYI   258
G. dehaani       ------------------   111
                  ** ********** ***
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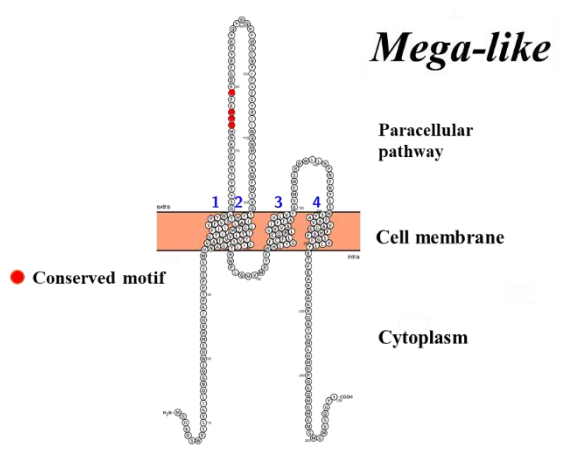
**Figure 10:** Amino acid alignment of mega-like proteins from *C. maenas* (accession no. GFXF01000913.1), *M. magister* (accession no. GHOI01010124.1), and *G. dehaani* (see Appendix I) using Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). (*) indicates identical AAs; (:) indicates AAs with similar properties; (.) indicate AAs with less similar properties. A grey under laid are AAs of a conserved motif, which can be found in vertebrate claudins and insect claudin-like SJPs.

A Protter (Protter–version 1.0, https://wlab.ethz.ch/protter) analysis of the *C. maenas* AA sequence predicted that the crab Mega-like protein has four transmembrane domains, two extracellular loops, one intracellular loop, and two intracellular regions. For *C. maenas* the transmembrane domains were predicted to be located at AA 38-60, AA 120-142, AA 162-184, and AA 199-221. (Fig. 11 A). The crab proteins contain, as also seen in the *D. melanogaster* AA sequence for Mega, a W-14X-GLW-2X-C-17X-C motif (Fig. 11 B). As suggested for the insect Mega, according to its secondary structure the crab Mega-like proteins from crabs are predicted to be claudin-like proteins (Fig. 2 A), sharing a typical conserved motif, GLW-X-X-C, which are found in vertebrate claudins. In addition, identified a Claudin-2 motif in the *C. maenas* Mega-like sequence (AA position 45-219) .

A




B

```
M.musculus Claudin 1      YSYAGDNIVTAQAIYEGLWMSCVSQSTGQIQCKVFDSLLNLNST
O.mykiss Claudin 4        SSFTGQNIVTAQETEEGLWMTCVVQSTGQQQCKSYESLLVLPSN
D.melanogaster Mega       YWIESYEETRASFKNMGLWQYCFKDFVYPKYAFLKQFTGCHNIF
C.maeans Mega-like        YWLQSYSYTYSDFKNMGLWEFCFDGFRYPKYQYDQKFMGCNYIF
M.magister Mega-like      YWLQSYSYTYSDFSNMGLWEFCFDGFRYPKYQYDHKFLGCNYIF
G.dehaani Mega-like       ----------------LWEFCFNGFRYPKYQYDYKFMGCNYIF
```

**Figure 11:** The Mega-like protein in decapod crabs. A) Protter analysis for the predicted secondary structure of Mega-like protein in *C. maenas* (258AA amino acids; accession no. GFXF01000913.1). (Protter–version 1.0). Red dots indicate the location of the conserved motif found in claudin-like proteins (GLW-X-X-C). B) Partial amino acid alignment of *Mus musculus* (house mouse) claudin 1 (accession no. AAH02003.1), *Oncorhynchus mykiss* (Rainbow trout) claudin 4 (accession no. XP_021481236.2), *D. melanogaster* (accession no. NP_569919.2), *C. maenas* (accession no. GFXF01000913.1), *M. magister* (accession no. GHOI01010124.1), and *G. dehaani* showing the conserved GLW-X-X-C motif (red under laid). Grey under laid are the conserved tryptophan and cytosine found in this position in Pancrustacea.

### *3.1.2 Kune-kune (Kune)*

Transcriptome mining employing the published Kune amino acid sequence from *D. melanogaster* (accession no. JV217000) allowed to identify a full open reading frame (ORF) for a Kune-like protein from *M. magister* (accession no. GHOI01010124; 223 AA; 39% identity and 52% similarity in AA the sequence to *D. melanogaster*) and a partial sequence from *C. maenas* (accession no. GFYV01332919). The obtained crab nucleotide sequences were then used to design crab-specific primers to obtain a 218 bp transcript coding for a Kune-like protein expressed in gills

of the freshwater crab *G. dehaani* (see Appendix I). Applying a tblastn search revealed between the obtained *M. magister* full open reading frame (ORF) of the Kune-like protein from and other true crabs, including *Callinectes sapidus* (accession no. GEID01103397.1), *Scylla olivacea* (accession no. GDRN01100958.1), *Eriocheir sinensis* (accession no. GFBL01087739.1), and *Hyas araneus* (accession no. HACH01026787.1) a ≥ 90% identity of the AA sequences.

As for *D. melanogaster* Kune and the *M. magister* Kune-like protein four transmembrane domains were predicted with two extracellular loops, one intracellular loop, and two intracellular regions (Fig. 13 A). For *M. magister* the transmembrane domains were predicted to be located at AA 12-37, AA 104-126, AA 138-159, and AA 175-198.

The crab proteins contain, as seen in the *D. melanogaster* AA sequence for Kune, a W-15X-GLW-2X-C motif (Fig. 13 B). As suggested for insect Mega and Kune, and according to the secondary structures, the crab Kune-like proteins are predicted to be claudin-like proteins (Fig. 2 B), sharing the typical conserved motif, GLW-X-X-C.

Also, similar for the findings for the crab Mega-like protein, the Motif-Finder (https://www.genome.jp/tools/motif/) identified a Claudin-2 motif in the *M. magister* Kune-like sequence (AA position 21-196) .

```
M. magister       MVVPPRERRTTAGWWAIGFHVLAFLLITIAMFTTYWLQAEKKAYGTAMDRIGLWTQCFRS        60
C. maenas         ------------------------------------------------------------        0

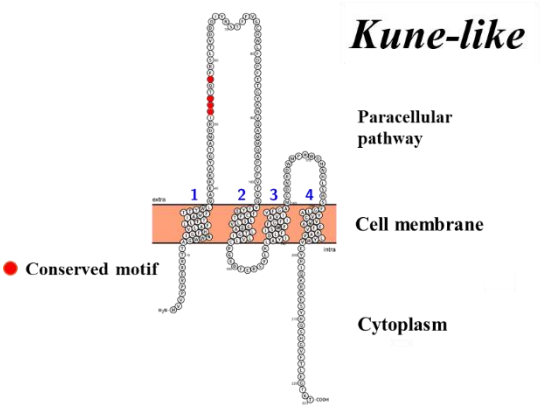
M. magister       LTVDDDIYRSTFFVGCRWLFDPFTTGYKNVQAMMQAPFFVTVQVFYTFCFTLSLVGTALT        120
C. maenas         -----------------------------------FFVTVQVFYTFCFTLSLIGTALT        35
G. dehaani        ------------------------------------------------------------        0
                                                    ***************** *****

M. magister       GILVLCPGEDFERSVLKLAYIDLFISWFFGFIAVIVFGAMGDNRDWMPHWDHNHLSWSFG        180
C. maenas.        GILVLCPGEDFERSVLKLAYIDLFISWFFGFIAVIVFGAMGDNRDWMPHWDHNHLSWSFG        95
G. dehaani        ----------------------FISWFFGFIAVIVFGAMGDNRDWMPHWEHNHLSWSFG        37
                  ************************************************:*********

M. magister       LAVVGVVAEFVAAVLYWVEYRIQKRKESYRQSHGVFTLEGTKT      223
C. maenas         LAVVGVVAEFVAAVLYWVEYRIQKRKESYRQSHGVFTLEGTKS      138
G. dehaani        LGVVGVVAEFVAAVLYLVEYRIQKRKESYRQSHGV--------      72
                  *.************** ******************
```

**Figure 12:** Amino acid alignment of Kune-like proteins from *M. magister* (accession no. GHOI01014120.1), *C. maeans* (accession no. GFYV01332919), and *G. dehaani* (see Appendix I) using Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). (*) indicates identical AAs; (:) indicates AAs with similar properties; (.) indicate AAs with less similar properties. A grey under laid are AAs of a conserved motif, which can be found in vertebrate claudins and insect claudin-like SJPs.

A




B

```
M.musculus Claudin 1      YSYAGDNIVTAQAIYEGLWMSCVSQSTGQIQCKVFDSLLNLNST
O.mykiss Claudin 4        SSFTGQNIVTAQETEEGLWMTCVVQSTGQQQCKSYESLLVLPSN
D.melanogaster Kune       WLVTDGRLQNPRFTNLGLWEVCFNNFQDIHRFFDNSFNGCLWVF
M.magister Kune-like      WLQAEKKAYGTAMDRIGLWTQCFRSLTVDDDIYRSTFFVGCRWL
E.sinensis Kune-like      WLQAEMKAYGTAMDRIGLWTQCFRSLTVTKDIYRERFFVGCRWL
```

**Figure 13:** The Kune-like protein in decapod crabs. A) Protter analysis for the predicted secondary structure of Kune-like protein in *M. magister* (223AA amino acids; accession no. GHOI01014120.1) (Protter–version 1.0). Red dots indicate the location of the conserved motif found in claudin-like proteins (GLW-X-X-C). B) Partial amino acid alignment of *Mus musculus* (house mouse) claudin 1 (accession no. AAH02003.1), *Oncorhynchus mykiss* (Rainbow trout) claudin 4 (accession no. XP_021481236.2), Kune of *D. melanogaster* (accession no. JV217000), *M. magister* and *Eriocheir sinensis* (accession no. GFBL01087739.1) showing the conserved GLW-X-X-C motif (red under laid). Grey under laid is the conserved tryptophan found in this position in Pancrustacea.

### *3.1.3 Contactin (Cont)*

Transcriptome mining employing the published Cont amino acid sequence from *D. melanogaster* (accession no. NP_649461.2) allowed to identify a partial Cont-like sequence from *M. magister* (accession no. GHOI01013052.1; 1302 AA; 44% identity and 61% similarity in the AA sequence to *D. melanogaster*) and *C. maeans* (accession no. GFYW01020902.1). The obtained crab nucleotide sequences coding for the Cont-like protein were then used to design crab-specific primers to obtain a 372 bp transcript coding for a Cont-like protein expressed in gills of the freshwater crab *G. dehaani* (see Appendix I). Applying a tblastn search revealed between the obtained *M. magister* full open reading frame (ORF) of the Cont-like protein and other true crabs, including *Callinectes sapidus* (accession no. GEID01054714.1), *Scylla olivacea* (accession no.

GDRN01099601.1), *Eriocheir sinensis* (accession no. GGQO01012410.1), and *Hyas araneus* (accession no. HACH01027487.1) a ≥ 86% identity of the AA sequences.

```
M.magister      MGLWAVVSVATMVVVAGRAQLFQNTLLLDPLLDEKVEKTCPTNWSTHQASCYKFIRSPVK          60
C.maenas        -----------------RAQMLQNTVLLDQ--ENNLENTCPTNWSIHKGSCYKFIRSPVK          41
                              ***::***:***   ::::*:******* *:.***********

M.magister      TRAQARAQCKAYGEGSSLVSVSNKEEHGFLTRYLHENDPLLRKWYTAGFEKTPGFWENEG          120
C.maenas        TRQQARAQCQAYGEGSDLVSVSNKDEHGFLTRYLHENDPLQRKWYTAGYQKTTGFWENEG          101
                ** ******:******.*******:*************** *******::** *******

M.magister      DGTSFQDMMEALLPEQTVTQQNKYLAYSYSVIHKEWGLLMVDGEEALLYVCEIPQANINN          180
C.maenas        DNSNFQDMMEALLPDQAATQEKVYLAYSYSFMHKEWGLLMVDGEDALLYVCEIPQANINN          161
                *.:.**********:*:.**:: *******.:************:***************

M.magister      IIDESVERDHEYGMVIQDPTKVPQSPMFVLQPDDTIFDLARREVINYISITCLAKGYPPP          240
C.maenas        IIDESVERNHEYGMVIQDPTKVPQSPVFVLQPKDTIFDLARREVVNYISITCLAKGYPPP          221
                ********:*****************:*****.***********:***************

M.magister      TYTWYKERYENEAIMSRPIDPLMDARFTVSGGTLIINNPKQVDDRGKYHCRATNKFGSIT          300
C.maenas        TYIWFKENYENEAIMSRAIDPLSDSRFTVSGGTLIINNPDQVEDRGKYHCRATNKFGSIT          281
                ** *:**.********* **** *:**************.**:*****************

M.magister      SESIQLSFAYVAEFNLKRSNEAGYQYWGKAIYCDPPQHFPDVRYYWARTYFPNLVEEDRR          360
C.maenas        SESIQFSFAYVAEFNLKRSDEVGNQYWGKAIYCDPPQHFPDVHYYWARTYFPNLVEEDRR          341
                *****:**************:*.* ******************:*****************

M.magister      TMVSYDGNLYFSYLDMIDTGQYSCNVMSTVSGIGKNGPLFNLEVVPHPNYQQLKFANNFP          420
C.maenas        TLVSHDGNLYFSYLDMIDNGLYSCNVMSTVSGIGKNGPFFNVDVVPHPNYQQLKFANNFP          401
                *:**:**************.* *****************:**::*****************

M.magister      KAFPEAPIRGEDVRLECIAFGYPVPSYNWTRRGGSLPRGAKLLNNNRVLVLPKVEPSDMG          480
C.maenas        KAFPEAPIRGQDVRLECIAFGYPVPAYNWTRRGGSLPRGAQLLSYNRILILPNVEPSDMG          461
                **********:***************:***************:**. **:*:**:*******

M.magister      DYICRVHNTRMAIQNGIALSIQAKPSFTIRLQDQFIAKGADLMWECESFGIPDVDYAWLR          540
C.maenas        DYICRAHNSRVAIENSIALSIQAKPSFTIRLQDQFIAKDADLMWECEAFGIPDVDYAWLR          521
                *****.**:*:**:*.**********************.********:*************

M.magister      NSELLETETLTVAEKERYEIVDSILKIKGVKESDEATYQCMATNQLGTSYSSAQLKVLKL          600
C.maenas        NSQPLKPETFTEDEKLRYELVDSIFKIKEVKESDEATYQCMATNQLGTTYSSAQLKVLKL          581
                **: *: **:*  ** ***:****:*** ********************:************

M.magister      APTFRKQPLETEMYGAEGKNVTIVCNPEAAPKPKFVWRQNGLIIGNGGRRRILRNGYLVI          660
C.maenas        APTFRKQPLETEMYAAEGKNVTIVCNPEAAPKPKFVWRQNGLMIGNGGRRRILRNGYLVI          641
                **************.*************************:*****************

M.magister      DPVSREDRGNYSCTAQNVYGTDTSYGWLEVLRKPEFYLSPPEEVVASKGDNITLECEAYT          720
C.maenas        DPVSREDRGNYSCMAENIYGSDVSYGRLEVLRKPEFYLSPPEQVIASKGDNITLECEAYT          701
                ************* *:*:**:*.*** ***************:*:***************

M.magister      DPLLDKSYIWRHNGLRIELDDTEYLRNLAYLQGYDYDYQRRMYNFYDNPYESSLYLKSRE          780
C.maenas        DPLLDKSYIWRHNGLRIELDDTEYLRHLAYLQGYDYDFQRRRYNLYDNPYESSLYLKRRE          761
                **************************:**********:*** **:************ **

M.magister      QEVYVAKRYPPYEKGYRAGHLRITLIQMEDAGVYECVAMTPVAKISISTKLTINGPPGPS          840
C.maenas        QEVYVSKRYPPYEKGFRAGHLRIPMIQMKDGGVYECVAKTPVAKISISTTVIIHGPPGPS          821
                *****:*********:******* :***:*.******* **********.: *:******

M.magister      GGVTALDLTSTKGRVQWTNGASNGKSILSYNIQTRTQWNRTWKLVARNITAELIDNTNGR          900
C.maenas        GGVAAVDLTSTRGRVQWTNGASNGRPILSYNIQTRTQRTQAWRTVAENIQAELIDNNNGR          881
                ***:*:*****:************: *********** .::*: **.** ******.***

M.magister      MEYQLEDKLSPWSAHEFRVQAVNILGEGQYSRSSPQANTRPDRPFTIPDKLGGGGGKTGD          960
C.maenas        MEYELADVLSPWSAHEFRVQAVNSLGLGQYSKSSPQANTRPDRPFTIPDKLGGGGGKTGD          941
                ***:* * *************** ** ****:****************************

M.magister      LTITWEPMPGEHQNAPGVYYKLFWRRSVLDPETEFQKIVLRDRGNVGMHVVDINPIKHFY         1020
```

```
C.maenas        LTITWEPMPGEHQNAPGIYYKLFWRRSVLDQETEFQKVVLRERGNVGMHVVDVNPIKYFY          1001
G.dehaani       ------------------------------------------------------------Y          1
                *****************:************ ******:***:**********:****:**

M.magister      TEYEVKIQAWNSIGPGPVSEPVTIYSAEDMPQVQPTEVSAFAHNATSLNITWQPIDPTRE          1080
C.maenas        TEYEVKIQAWNSIGPGPISEPVTIFSAEDMPQVQPTEVSAFAHNATSLNVTWQPINPTRE          1061
G.dehaani       TQYEVKIQAWNSQGPGPISEPVEVFTAEDMPQVQPTEVSAFAHNATSLNVTWQPIEPTRE          61
                *:********** ****:**** :::************************:*****:****

M.magister      NIRGKLVGYRIKYWRRMDNETDHIIKLQRGIEPHGLIVGLVPNTFYWVRVMAYNQAGSGP          1140
C.maenas        NIRGKLVGYRIKYWRRMDNETDHIIKLQRGTEPHGLIVGLVPNTFYWVRVMAYNEAGSGP          1121
G.dehaani       NIRGKLVGYRIKYWRRKDSETDHVIKLQRGTEPHGLIVGLVPNTFYWVRVMAYNQAGSGP          121
                *************** *.****:****** ***********************:*****

M.magister      ESERFLERTWRQPPLMPPNAVKVYPINPSTIRVTWRGVTPTPMEEPLIGYKIKVWESDQD          1200
C.maenas        ESERFLERTWRHPPLMPPNAVQVYPIDPGTIRVTWRGVTPTPMEEPLIGYKVKVWESDQD          1181
G.dehaani       ESE-------------------------------------------------------          124
                ***********:*********:****:*.**********************:********

M.magister      FTTANDTFVYIGSPLEAYISDLAPGKSYMLRVLAFSRGGDGKKSSPPWKFQMGDTDVLRG          1260
C.maenas        FTTANDTYVFIGSPLEAYITDLSPGKSYMLRVLAYSRGGDGKKSSPPWKFQMGDTDVLRG          1241
G.dehaani       -------------------------------------------------------------          124
                *******:*:*********:**:***********:************************

M.magister      MAPGTSPDMLLNTGVLVTFLVGFLTPILRSRFFHHHHHHHHNH                            1302
C.maenas        TAPSTFVETLLNSALLVTFAVGFAS-----------------                             1266
G.dehaani       ------------------------------------------                             124
                **.*  : ***:.:**** *** :
```

**Figure 14:** Amino acid alignment of Cont-like proteins from *M. magister* (accession no. GHOI01013052), *C. maeans* (accession no. GFYW01020902.1), and *G. dehaani* (see Appendix I) using Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). (*) indicates identical AAs; (:) indicates AAs with similar properties; (.) indicate AAs with less similar properties. A grey under laid are AAs of a conserved motif, which can be found in vertebrate claudins and insect claudin-like SJPs.

A Protter analysis (Protter–version 1.00) revealed that the crab Cont-like protein contains a signal peptide (AA 1-19), one transmembrane domain (AA 1269-1288), one large extracellular region, and a short intracellular region (Fig. 15).

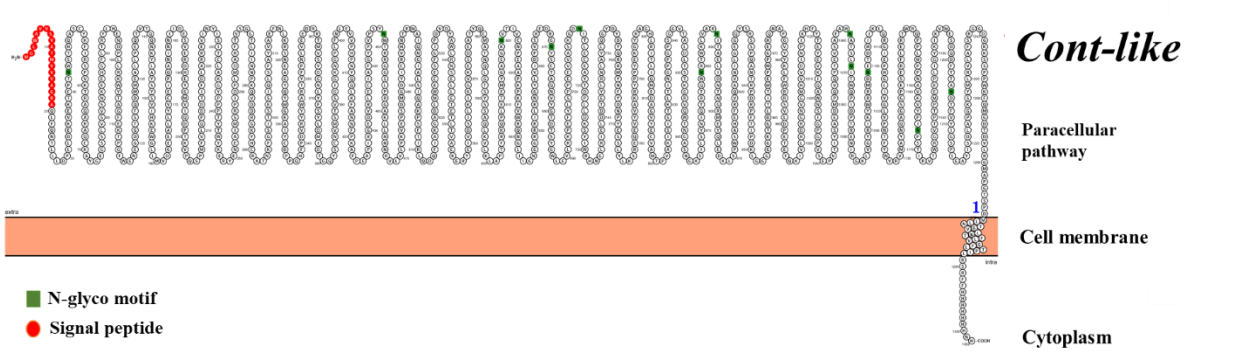



**Figure 15:** Predicted secondary structure of the Cont-like protein in *M. magister* (accession no. GHOI01013052.1) consisting of 1302 amino acids. Green dots indicate N-glycosylation motifs. At the N-terminus a signal-peptide was identified red (Protter–version 1.0).

### *3.1.4 Neurexin-IV (Nrx-IV)*

Transcriptome mining employing the published Nrx-IV amino acid sequence from *D. melanogaster* (accession no. CAA60383.1) identified Nrx-IV-like sequences from *M. magister* (accession no. GHOI01007751.1; 1264 AAs; 54% identity and 72% similarity in the AA sequence to *D. melanogaster* (accession no. JV209786.1), and *C. maeans* (accession no. GFXF01000027.1). The obtained crab nucleotide sequences coding for a Nrx-IV-like protein were then used to design crab-specific primers to obtain a 169 bp transcript coding for a Nrx-IV-like protein expressed in gills of the freshwater crab *G. dehaani* (see Appendix I).

Applying a tblastn search revealed between the obtained *M. magister* full ORF of the Nrx-like protein and other true crabs, including *Callinectes sapidus* (accession no. GEID01161579.1), *Scylla paramamosain* (accession no. GIXE01007421.1), *Eriocheir sinensis* (accession no. GFBK01009212.1), and *Hemigrapsus takanoi* (accession no. GKPI01389235.1) a ≥ 88% identity of the AA sequences.

```
M.magister      MEGPILTLITLLLGPILVLSDYCEYPLVEESRLTASSELVTREADKARLYETTAWTARSA          60
C.maeans        ------------------------------------------------------------          0

M.magister      DYLQYIEADMGDIKNVTAIATQGREDSQEFVTAYTLEYSRDGVHYSIVMGLKGNIMAFPG         120
C.maeans        ------------------------------------------------------------          0

M.magister      NKDGDTVTTNLFETPIIARYIRIKPTRWRDRISMRLEIYGCQYMPETVSFNGNAMVVMEL         180
C.maeans        ------------------------------------------------------------          0
```

```
M.magister      RKFPIASLRDHIRFRFRTKEPDAMVMYARGTQGDYVALQLVQNRMVLNVNLGSRLMTSLS          240
C.maeans        ------------------------------------------------------------          0

M.magister      VGSLLDDNSWHDVEIRREQRNITFLVDRVRVDDIILGDFKRLDLNKELYLGGVPNLQLGM          300
C.maeans        ------------------------------------------------------------          0

M.magister      KARVNFTGCLENLFINGTAIIPEMREADDYYSYYNSRPKYLTINTGRVCEFGISADQTMT          360
C.maeans        ------------------------------------------------------------          0

M.magister      FTSRKSHLKYPAFEDQRKINVSVEFRTYEEEGVLIHHKFSTRGFFTLYLEEGKIKVKIEA          420
C.maeans        ------------------------------------------------------------          0

M.magister      EGTPGVVVLDNFDVSYNDGQWRRVMFIIMQNRMELSVDGVPMQTVRIISVISGKFFFIGG          480
C.maeans        ------------------------------------------------------------          0

M.magister      GVYGAPGFMGCLRKISVVGYMQKPKDEEIKYPEGIVRAACQIMDRCNPNPCEHRGRCKQN          540
C.maeans        -----------------VGYMQTPKDEEIFHPAGIVRAACQIMDRCNPNPCEHRGRCKQT          43
                      *****.****** :* *************************.

M.magister      SQEFFCDCTGTGYSGAVCHAPLNPISCAAYGIQNPGVKRAEIYIDIDGSGPLVPFPVTCE          600
C.maeans        SQEFICDCTGTGYSGAICHTPLNPISCAAYGIQNPGVKRAEIYIDIDGSGPLVPFPVTCE          103
                ****:***********:**:****************************************

M.magister      FYNDGQVHSYLSHKHEKLTTVDGFEKRGSYIQNIIYDAGMEQIEIFVNRSAKCRQRIHFE          660
C.maeans        FYNDGQVHSYLSHKHEKLTTVDGFEKRGSYIQNIIYDAGMEQIEIFVNRSARCRQRIHFE          163
                ************************************************:********

M.magister      CLKAKLFNSPSPEDQDFLPYTWWVSRTNQPMDYWGGSLPGSRKCECGLMGTCVTDKWCNC          720
C.maeans        CLKAKLFNSPSREDEEFLPYTWWVSRTNQPMDYWGGSLPGSRKCECGLMGTCVTDKWCNC          223
                ********** **::**********************************************

M.magister      DAGLESWLSDSGELTVKEHLPVRQLRIGDTGTPLDGKKVRFTLGPLICEGDHVYDNTVTF          780
C.maeans        DAGLESWVSDSGELTVKEHLPVRQLRIGDTGSPLDGKKVRYTLGPLVCEGDHVYDNTVTF          283
                *******:**********************:********:*****:**************

M.magister      RKADGTINLPRFDMGHSGDIFFEFKTTTRDGCLIHAHGVSDYIKISILGGIELQFQYQAG          840
C.maeans        RLSDATITLPRFDMGHSGDIFFEFKTTTRDGCLIHAKGPTDYIKISIVGGIELQFEYQAG          343
                * :*.**.*****************************:* :*******:*******:****

M.magister      AGPMSVSVETSNVLNDDRWHSVLVERNRKEARMIVDGGRKAVMAEPPGPVRAIHLDSDFV          900
C.maeans        AGPMSVSVETSNTLNDDTWHSVLVERNRKEARMIVDGGRKSVMAEPRGPVRAIHLESDFV          403
                ************.**** ***********************:***** ********:****

M.magister      VGATTDFRDGYVGCIRALILNGELQDLRGRAERGLYGVQEGCLGKCHSNPCLNNGTCHEG          960
C.maeans        VGATLDFRDGFVGCIRALILNGVLQDLRSRAERGMYGVVPGCVGKCQSSPCLNNGTCHEG          463
                **** *****:*********** *****.*****:***  **:***:*.************

M.magister      YSTFECDCRWTAFKGPICADEIGIKMLTDTMVRYEIPGTYKTTIAERIRVGFTTTNPRGF          1020
C.maeans        YATYECDCRWTAFKGPICADEIGIKMLTDTMVRYEIPGTYKTTIAERIRVGFTTTNPRGF          523
                *:*:**********************************************************

M.magister      LIGLHSNLTGEYLTLAISNSGHLKVTFDFGFERHEKVYGKRTFHEGQNHDVKLYRSDSGR          1080
C.maeans        LIGLHSNLTGEYLTLAISNSGHLKVTFDFGFERHEKVYGKRTFHEGQNHDVKLFRSDSGR          583
                ********************************************************:******

M.magister      RLTMQVDNYEPVSWTFDVKGSADAQFNNIQYVYIGKNESMAEGFVGCISRVEFDDIYPLK          1140
C.maeans        RLTMQVDNYEPVSWTFDVKGSADAQFNNIQYVYIGKNETMAEGFVGCISRVEFDDIYPLK          643
G.dehaani       ----------------------------FQYVYIGKNESMAEGFVGCISRVQFDDIYPLK          32
                ****************************:*********:************:********

M.magister      FYFQQDRPATVIAESTSSVFEDYCGIEPIRYAEEKAETRPPPEVSKEVLMELYSDNSAVL          1200
C.maeans        FYFQQDRPASVIAESTSSVFEDYCGIEPIRYPEEEAETRPPPEVSEEVLMELYADNSAVL          703
G.dehaani       FYFQQDRPHEITAESTSSIFEDYC------------------------------------          56
                ********  : ******:************ **:**********:*******:******
```

```
M.magister      GGVLGIIFLALLCMGFLIGRYMARHKGDYRTHEADGADVAPDADWAVQHATTGPQVKKNT   1260
C.maeans        GGVLGIIFLALLCMGFLIGRYMARHKGDYRTHEADGADVAPDADWAVQHATTGPQVKKNT   763
G.dehaani       ------------------------------------------------------------   56
                ************************************************************

M.magister      EMYI                                                           1264
C.maeans        EMYI                                                           767
G.dehaani       ----                                                           56
                ****
```

**Figure 16:** Amino acid alignment of Nrx-IV-like proteins from *M. magister* (accession no. GHOI01007751.1), *C. maeans* (accession no. GFXF01000027.1), and *G. dehaani* (see Appendix I) using Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). (*) indicates identical AAs; (:) indicates AAs with similar properties; (.) indicate AAs with less similar properties.

The predicted secondary structure (Protter–version 1.0) of the Nrx-IV-like protein of *M. magister* has one transmembrane domain (AA 1198-1218), one intracellular region, and one large extracellular region that includes a signal peptide (AA 1-20) (Fig. 17).

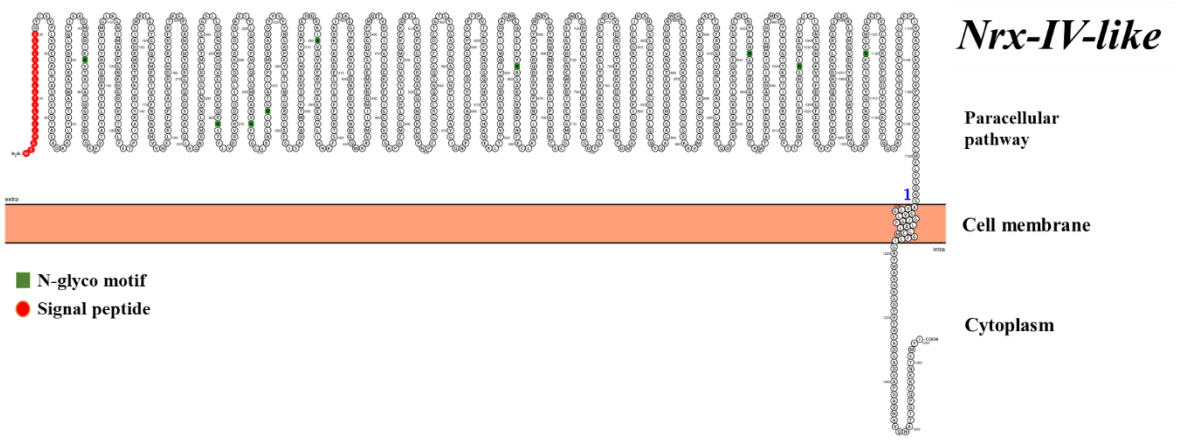



**Figure 17:** The predicted secondary structure of the Nrx-IV-like protein in *M. magister*, consisting of 1264 amino acids. Green dots indicating N-glycosylation motifs. At the N-terminus a signal-peptide was identified red (Protter–version 1.0).

### *3.1.5 Würmchen1 (Wrm1)*

Transcriptome mining employing the published Wrm1 amino acid sequence from *D. melanogaster* (accession no. NP_648105.1) identified a Wrm1-like sequences from *C. maenas* (accession no. GFYV01022712.1; 85 AA; 43% identity and 58% similarity in the AA the sequence to *D. melanogaster* (accession no. JV209786.1) and *M. magister* (accession no. GHOI01037666.1). The obtained crab nucleotide sequences coding for a Wrm1-like protein were

then used to design crab-specific primers to obtain a 112 bp transcript coding both for a Wrm1-like protein expressed in gills of the freshwater crab *G. dehaani* (Fig. 18) (Appendix I).
Applying a tblastn search revealed between the obtained *C. maenas* full open reading frame (ORF) of the Wrm-like protein and other true crabs, including *Callinectes sapidus* (accession no. GEID01111707.1), *Scylla olivacea* (accession no. GDRN01078369.1), *Eriocheir sinensis* (accession no. GEFT01036698.1 ), and *Hyas araneus* (accession no. HACH01022917) a ≥ 86% identity of the AA sequences.

```
C.maenas        MPAFNNTLPEFNNQFGIKAEYFNYTPFYVGITFCTILAIVLIMVNWICSCCHEHSEYWND          60
M.magister      MPAFNNTLPEFNNRFGIKPEYFNYTPFYAGIIICTLLATVLIMVNWICSCCHEHSKYWND          60
G.dehaani       ------------------PEFYNYTPFYVGITICTIIGIILIMVNWICSCCHEH------          36
                *************:**** *::******.** :**::. :***************:****

C.maenas        PDTGNRFASLIFVRKAKQRPLDALY       85
M.magister      PDTGNRFASLIFVRKAKQRPLDALY       85
G.dehaani       -------------------------       36
                *************************
```

**Figure 18:** Amino acid alignment of Wrm1-like proteins from *C. maenas* (accession no. GFYV01022712.1), *M. magister* (accession no. GHOI01037666.1), and *G. dehaani* (see Appendix I) using Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). (*) indicates identical AAs; (:) indicates AAs with similar properties; (.) indicate AAs with less similar properties.

The predicted secondary structure of Wrm1-like in *C. maenas* (accession no. GFYV01022712.1) has one predicted extracellular domain starting on the N-terminus, followed by one transmembrane domain (AA 27-48), and one intracellular region (Fig. 19).

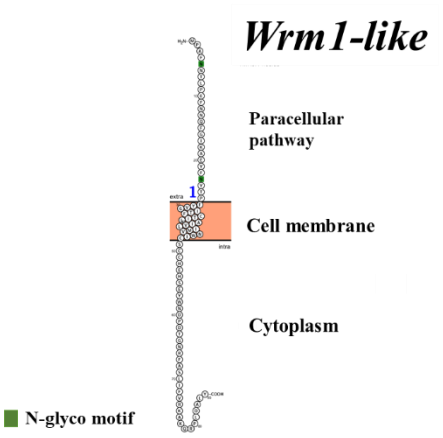



**Figure 19:** The predicted secondary structure of the Wrm1-like protein in *C. maeans*, consisting of 85 amino acids. Green dots indicating N-glycosylation motifs (Protter–version 1.0).

Note, the Wrm1-like protein expressed in crabs is considerably shorter (85 *vs.* 160 AAs) when compared to the Wrm1 proteins in insects. Additionally, the extracellular domain does not seem to be conserved (Fig. 5). This leads to the conclusion that the newly discovered protein may have a different function when compared to the insect Wrm1. We henceforth name this potential SJP Würmchen1-like.

```
S.olivacea          MPPYNNTLPA-FNNQFGIKPEFYNYTPFYIGITFCTILGIILIMVNWICSCCHEHSKYWN      59
C.maenas            MPAFNNTLPE-FNNQFGIKAEYFNYTPFYVGITFCTILAIVLIMVNWICSCCHEHSEYWN      59
M.magister          MPAFNNTLPE-FNNRFGIKPEYFNYTPFYAGIIICTLLATVLIMVNWICSCCHEHSKYWN      59
D.melanogaster      MSTIEEERKAYEKNPYFTGHIYGNFSPFYVTIAICTVVLGTIIILNIILGCCSKHRKYWQ      60
M.domestica         MST-IDERTAYKNNPYFTGHIYGNFSPFYVTIAVCTVVFSAIILINIILGCCSKHRKYWQ      59
                    *    :      :* :     : *::***  * .**::   :*::* * .** :* :**:

S.olivacea          DPDTGNRFASLIFVRKAKQRP-LDALY---------------------------------      85
C.maenas            DPDTGNRFASLIFVRKAKQRP-LDALY---------------------------------      85
M.magister          DPDTGNRFASLIFVRKAKQRP-LDALY---------------------------------      85
D.melanogaster      DRHTGNRWLVSIWSATPHNQPPLDFTELKDASYFQRFHPTTHQQVFPDDVVIGVDDLEPV      120
M.domestica         DRHTGNRWLVSIWSATPHNQPPLDLTELKDASYFQKLQPA--QRIFPEDIEAQGEEV-P-      115
                    * .****:   *:  . :::* **

S.olivacea          ---------------------------------------------                  85
C.maenas            ---------------------------------------------                  85
M.magister          ---------------------------------------------                  85
D.melanogaster      HSAHHHHHQQQHQRPPRPEGRTLHQQ-RQREEYVELQKR-ESDI                   162
M.domestica         --IHQYQQQQHQSRPPRPEGRSLHQQQRHREEYVELQTKRESDI                   157
```

**Figure 20:** Amino acid sequence alignment of insect Wrm1 (*D. melanogaster* (XP_001654796.1) and *Musca domestica* (XP_005180352.1) and the Wrm-like protein from *Scylla olivacea* (GDRN01078369.1), *C. maenas* (GFYV01022712.1), and *M. magister* (GHOI01037666.1). Yellow background indicates the predicted transmembrane domain in the crab sequences. Blue background indicates the predicted transmembrane domain in the insect sequences (Protter–version 1.0)

*3.1.6 Gliotactin (Gli)*

Transcriptome mining employing the published Gli amino acid sequence from *Aedes aegypti* (accession no. KX823345.1) allowed to identify a partial Gli-like sequences from *M. magister* (accession no. GHOI01016740.1; 1010 AAs; 48% identity and 64% similarity in the AA sequence to *D. melanogaster* (accession no. AAC41579) and *C. maeans* (accession no. GBXE01097000.1). The obtained crab nucleotide sequences coding for the Gli-like protein were then used to design crab-specific primers to obtain a 606 bp transcript coding for a Gli-like protein expressed in gills of the freshwater crab *G. dehaani* (see Appendix I).

Applying a tblastn search revealed between the obtained *M. magister* full open reading frame (ORF) of the Gli-like protein and other true crabs, including *Callinectes sapidus* (accession no. GEID01161579.1), *Scylla paramamosain* (accession no. GIXE01007421.1), *Eriocheir sinensis* (accession no. GFBK01009212.1), and *Hemigrapsus takanoi* (accession no. GKPI01389235.1) a ≥ 88% identity of the AA sequences.

```
M.magister      VVVVTVEAQFRNDRDHETLRDDYWQTHYRNQGYYTTPSSVRTQYNTGDTYYGTDRRYDTR          60
C.maenas        VVVTAVEGQFRNDRDHDSLRDDYWQTHYRNQGYYTTPPPVRTQYNSGDTYYGTDRRYDTR          60
                ***.:**.********::******************  ******:**************

M.magister      YDDPSQTRGFDNRFQDQSFRYNENPKLSGNYLGRGRYDARGRERPGA-RPGDDLWDMRLG          119
C.maenas        YDDPSQTRGFDNRYQDQSFRYSENPRLSGNYLGRGRYDARGRERPGGARGGENDWDYRLG          120
                *************:*******.***:**********************. * *:: ** ***

M.magister      VLDIWRPDLQGELRPSEEPGLTLPLADIFVTTDSGKVQGFYVYLYDKPGVRHNERPVNKP          179
C.maenas        VQEMWRPDLQGEFRPSEEPGFTLPLADIFVTTDSGKVQGFYVYLYDKPGVRHNDRPVNKP          180
                * ::********:*******:**********************************:******

M.magister      IHQQRHIMNVSTFLGIPYAKPPLLEGRFKPPRHVPNWYSTWQALDYQSACPQNLKYIGAS          239
C.maenas        IHQQRHIMNVSTFLGIPYAKPPLLEGRFKPPRHVPNWYSTWQALDFQSACPQNLKYIGAS          240
G.dehaani       ******************************PRHVPNWYSTWQALDYQSACPQNLKYIGAS          30
                                              ***************:**************

M.magister      NGIRNGIHEDCLYLNIFSPSVSINVNNMYPVLFYIHGGDLDHGASNQFPGHMLAAFGEVV          299
C.maenas        NGIRNGIHEDCLYLNVFTPSVSINVNNMYPVLFYIHGGDLDHGASNQFPGHMLAAFGEVV          300
G.dehaani       NGIRNGIHEDCLYINIFSPSVSITVNDMYPVLFYVHGGDLYHGASNQFPGHMLAAWGQVV          90
                *************:*:*:*****.**:*******:***** **************:*:**

M.magister      VVTINYRLGALGFLSTGDANSPGNYGLMDMAMALEWVYTNIRFFNGDRNKITVFGPGAGG          359
C.maenas        VVTFNYRLGALGFLSTGDANSPGNYGLMDMAMALEWVYTNIRFFNGDRNKITVFGPGAGG          360
G.dehaani       VVTFNYRLGALGFLSTGDANSPGNYGLTDMAMALKWVHTNIRFFNGDRNRITVFGPGAGG          150
                ***:*********************** ******:**:***********:**********

M.magister      AMAGLLAVLPRTKDWVSQVIAQSGSALADWAVLDDVYRVQNTSRVYGLEVGCTVESSYKL          419
C.maenas        AMAGVLAVLPRTKDYVSQVIAQSGSPLADWAVLDDVYRVQNTSRVYGMEVGCTVESSYKL          420
G.dehaani       AMAGLLAVLPRTKDYVNQVIAMSGSPLAEWAALDDVYRVQNTSRVYGLEVGC--------          202
                ****:**********:*.**** *** **:**.***************:************

M.magister      LQCLNRRSYEELSTAPITPDVGTFAWGPAVDTNFTVPGDEWYEEWEEKDWHVVPEIPLNL          479
C.maenas        LQCLNRRSYEELSTAPITPDVGTFAWAPALDTNFTVPGDDWYEEWKEKDWHIVPEMPLHL          480
                *************************.**:*********:*****:*****:***:**:*
```

```
M.magister      YLKNHHHPNLRYLTGVNRDAAANFVFNDNRLVRSRHVVTEEFFNERVKEWVRHYNYSLNL   539
C.maenas        YQRNHHHRNLRYLTGVNRDAAANFVFNDNRLVRNRHEVTEEFFNQRIKEWVRHYNYSLNL   540
                * :**** ************************.** *******:*:**************

M.magister      EGAYDAIKWMYTYGPDPHNTTHIREEYVNMLSDALYKSAVDQIVKVLVNSTNKQRVPTYY   599
C.maenas        EGAYDAIKWMYTYGPDPHNTTHIREQYVHMLSDALYKADVDQIVKVLVNSTNKQSVPTYY   600
                *************************:**:********: *************** *****

M.magister      YLLNTTVEALKLPLWREIPNDNEYYFISGAPFMDPDFYPEAMRVSREQWSEGDRNISQFM   659
C.maenas        YLLNTTVEALKLPFWREIPNDIEYYFISGAPFMDPDFYPEATRVSREQWGEGDRNISEFM   660
                *************:******* ****************** *******.*******:**

M.magister      MKTLANFAKWGNPTQTQVEVLKVNWEQVLEGNLKYLSINTTFNTSMHYNYRQPYNTFWTT   719
C.maenas        MKTIANFAKWGNPTQTQLEVLKVKWDEVLEGNLRYLSINTTFNTTMHYNYRQPYNTFWTT   720
                ***:**************:*****:*::******:**********:***************

M.magister      YMPQVIREWVPTVPPPLNPWTQMSQPIVAAFYGSLMACVVLLVILFVCCGLWKSAKRQRN   779
C.maenas        YMPQVIREWVPTVPPPLNPWTQMSQPIVTAFYGSLMACVVLLVVLFVCCGLWKSAKRQRN   780
                ****************************:**************:****************

M.magister      KALDDLQYISSDHLEPPHDQDDYKVREYLDRLSNHQLSNGHTSSRPITPTSRPITPNTVT   839
C.maenas        KALDDLQYISSDHLEPPDDQDDYKVRDYLDRLSNPQLSNGHSQSRPITPTSRPITPNTIT   840
                *****************.********:******* ******:.***************:*

M.magister      TEAPELSEAGTLKRGNGNQESYRHNHHSMAKSVDQLNHPRYQPNYNNDPQPAQVHLSRST   899
C.maenas        TEAPELSEAGTLKRGNGNHEAYRHNHHTMAKSVDQLNHPRYQPSINHNHPSTQVHHSRST   900
                ******************:*:******:***************. *::   :*** ****

M.magister      DNILESYPRSYITPDMGAQLNGHTHQP--QQRKNNNNNNHILAMTPPQQMRPITPAASEV   957
C.maenas        DNIMESYPRAYITPDMGPQLNGHMHQAQPQERKKHNYINNNINMTPPQQMRPITPA-SEI   959
                ***:*****:******* ***** **   *:**::*  *: : ************* **:

M.magister      HSEPLRRPSYDSAFATQPLVCEELEKPPRTPVPMIRTSVATITSTTGLLHTEL          1010
C.maenas        HSEPQRRPSYDSAFSTQPLVCQELEKPPRTPVPALRTSVATITSTTGLLHTEL          1012
                **** *********:******:*********** :******************
```

**Figure 21:** Amino acid alignment of Gli-like proteins from *M. magister* (accession no GHOI01016740.1), *C. maeans* (accession no. GBXE01097000.1), and *G. dehaani* (see Appendix I) using Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). (*) indicates identical AAs; (:) indicates AAs with similar properties; (.) indicate AAs with less similar properties.

Since the full ORF for the Gli-like protein has not been published for either *M. magister* or *C. maenas* we used date available in the mud crab *Scylla paramamosain* (accession no. GIXE01001464.1) to analyse the ORF (1037 AA). The AA sequence from this crab shares 91% identity to the 1010 AA long counterpart from *M. magister*. A Protter analysis revealed that the crab Gli-like protein contains a signal peptide (AA 1- 27) at the N-terminus as part of an extended extracellular domain of the protein. A single transmembrane domain (AA 773-796) connects the sequence to the intracellular domain (Fig. 22).

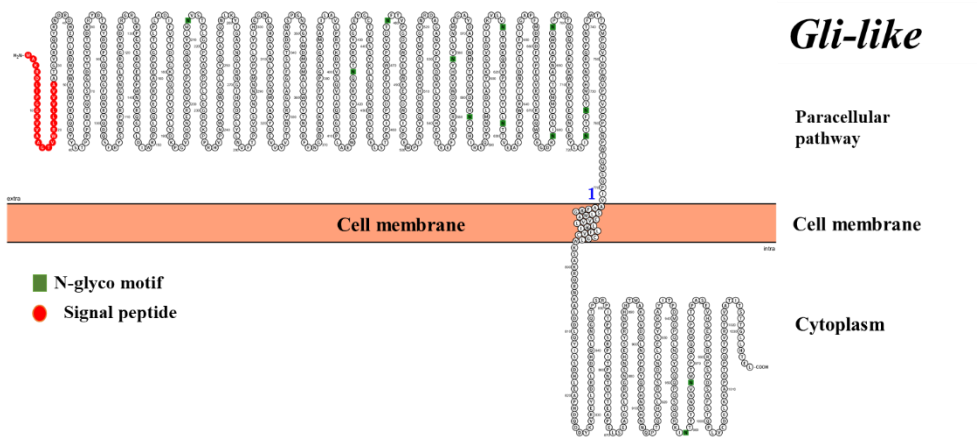



**Figure 22:** The predicted secondary structure of the Gli-like protein in *Scylla paramamosain* (accession no. GIXE01001464.1), consisting of 1037 amino acids. Green dots indicating N-glycosylation motifs. At the N-terminus a signal-peptide was identified red (Protter–version 1.0).

### *3.1.7 Mesh*

Transcriptome mining employing the published Mesh amino acid sequence from *D. melanogaster* identified Mesh-like sequences from *C. maenas* (accession no. GBXE01116262.1; 1308 AA; 51% identity and 65% similarity in AA the sequence to *D. melanogaster* Mesh isoform D (accession no. NP_001163782.1), which potentially represents the full ORF. GenBank had no entrance for a Mesh-like protein for the Dungeness crab. Employing degenerate primers allowed for the sequence and identification of a 370 bp long transcript coding for a Mesh-like protein in *M. magister* (Appendix II). The partial AA sequence showed 75% identity to the corresponding sequence in *C. maenas*. So far no Mesh-like sequence could be identified in *G. dehaani.*

Applying a tblastn search revealed between the obtained *C. maenas* (potentially) full ORF of the Mesh-like protein and other true crabs, including *Portunus sanguinolentus* (accession no. GFZC01028918.1), *Gecarcoidea lalandii* (accession no. GIKV01017064.1), *Eriocheir sinensis* (accession no. GGQO01012360.1), and *Hemigrapsus takanoi* (accession no. GKPI01193518.1) a ≥ 83% identity of the AA sequences.

```
C.maenas        MACRMTQATQAAGPPTKNEVDQLLQQQQQEERLMNVASILEKEGGVYDPMKSRVAPSADG          60
M.magister      ------------------------------------------------------------          0

C.maenas        GPPRVKLGNSRVKRNVGNDPMMSRMAPQQWQPDLGPYAITSERLKEIRAQKMYPYFDSDE         120
M.magister      ------------------------------------------------------------          0
```

```
C.maenas        RGGNGDLEGNINTQNTQVNKQLNFLLPFFGFGLNYTWVSLNGYLGFSDAPFNWANYPLSF   180
M.magister      ------------------------------------------------------------   0

C.maenas        PVQDWPNRPDPSFIGPFYSKCNIGELRPGDDNSRRPGVYWRMARDLPSREDQFSVELRER   240
M.magister      ------------------------------------------------------------   0

C.maenas        LMWDIREGMVGTAIFKPKHALMVTWKNVTFAGGSVNTDAKFVTNTFQLVVATDEIRTYTI   300
M.magister      ------------------------------------------------------------   0

C.maenas        FNYDYMRWTSHTEAGGSTDEGQGGVPAYVGFNAGNGTRSYEYTPYSQKLYIRDLAVAGNA   360
M.magister      ------------------------------------------------------------   0

C.maenas        NGFPGRHIFRVDEKILAGCCRREEELKDYPLTFSPEQGNMLGGALVNLTGPCFKTSYRLT   420
M.magister      ------------------------------------------------------------   0

C.maenas        CQFDTTAVEGHILDDNRATCIMPQLYVSGYVDFSISINNGPYYWKGKFFVETPLTAPEGV   480
M.magister      ------------------------------------------------------------   0

C.maenas        WFKDDAYQHHSPETLSIEWLAGNLTMNRDASAMVSLYGYREISIRPEIVYIDILAEGVPN   540
M.magister      ------------------------------------------------------------   0

C.maenas        LGYYTLTTADYLNRDNRELLDLEMGIVMINLTVPEPIYGLKKSTTVWSRPIPLAWYFRFQ   600
M.magister      ------------------------------------------------------------   0

C.maenas        WERLFGTSWPTAMCDRWIENDRNLRNFAYEVERCPCLLQQAVADKGRFLPDFSCDKDGNT   660
M.magister      ------------------------------------------------------------   0

C.maenas        QCDYHFGAIHCVRTALPNEDGAGQQCCYDRDGYLMMTADKMWGGNPHRAHNLGKTPFDEA   720
M.magister      ------------------------------------------------------------   0

C.maenas        NKVPSLSHWYHDVVPFYTCCKWQGEQSPGCVTYRFERRASQDCVGYQPPTAATVFGDPHI   780
M.magister      ------------------------------------------------------------   0

C.maenas        YTFDDLPYTFNGKGEFVLARVDSVRHKLDVQARFEQISPNYLQYAKGSMLTAVAARDNIS   840
M.magister      ------------------------------------------------------------   0

C.maenas        SVVEVRQRPIDAIWRYHLDVIVDGQRVYFDRYSQKIQQFRECVVYTPSNILNQSHVVIMF   900
M.magister      ------------------------------------------------------------   0

C.maenas        ASGAGVEVMENRGFLGTRVYLPLSFANITRGLFGNWTFLMEDDFTKPDGTEGPAAEASNM   960
M.magister      --------------------------------LWDWTFDPEDDFTLPDGSLGPAAEASNM   28
                                                : :***  ***** ***: *********

C.maenas        KAVH-TYGMEWVLDDKEREYVGRSLFYHDNYRSSNYYYDADFVPEMVIDPVMSINATTTS   1019
M.magister      LVVHRDFGMHWVLDDKEKTDVGRSLFNHDNYRSSNYFYDPIFEPEWIIDPVLSINATTKS   88
                 .**  :**.*******:  ****** *********:**  * ** :****:******.*

C.maenas        EEVKRACGDSYQCTFDFVVTLRRDFADMTKYYQDQFVNIKQIGLVNVVSCGALPTPPNGR   1079
M.magister      DEIKRVCGDSYQCHFDYVVTLRRDFADITKYYQDQ-------------------------   123
                :*:**.******* **:**********:*******

C.maenas        KSTFNFLSGAEVKFDCDPGYVLLGEQRRWCYASGDWNWPEDGEATCVTEAEYLVMQGGIT   1139
M.magister      ------------------------------------------------------------   123
```

```
C.maenas        AGTVLAVLLPVLIALLCFTSYLRNKERYDAQTYAAVLPRRTKAPAPSRPVISRPMGPVKT   1199
M.magister      ------------------------------------------------------------   123

C.maenas        ADYSPVTHREVFKGSPPPQKLLTEAPLGRAYDSSSDLPETPSSHSLSDSDSYTYPGNSPA   1259
M.magister      ------------------------------------------------------------   123

C.maenas        LDAIGCRPMSIPKTFDGVYDTHEPVNNKPVVFQNVLWDLDSEPQKESKV              1308
M.magister      -------------------------------------------------              123
```

**Figure 23:** Amino acid alignment of Mesh-like proteins from *C. maenas* (accession no GBXE01116262.1) and *M. magister* (see Appendix II) using Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). (*) indicates identical AAs; (:) indicates AAs with similar properties; (.) indicate AAs with less similar properties.

The predicted secondary structure of the Mesh-like protein in *C. maenas* has one large predicted extracellular domain starting on the N-terminus, followed by one transmembrane domain (AA 1132-1157), and one intracellular region (Fig. 24).

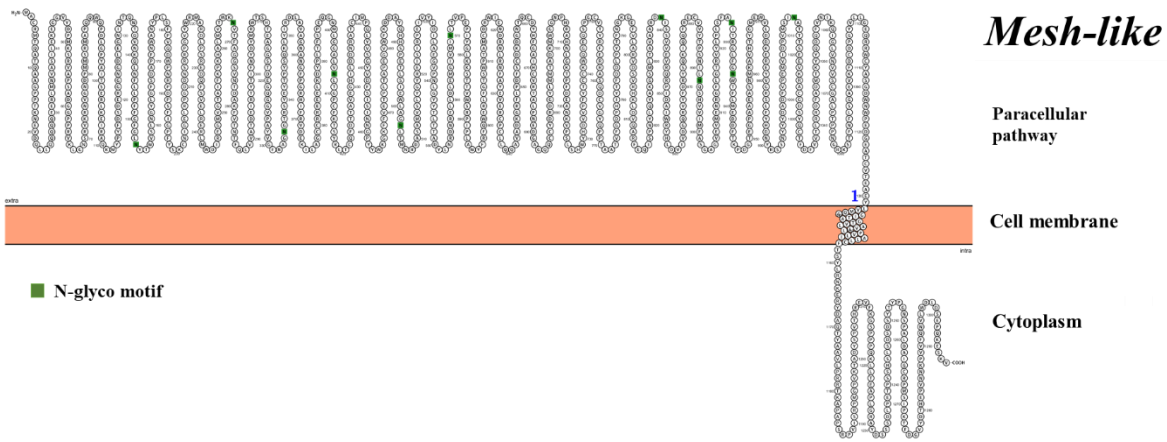



**Figure 24:** The predicted secondary structure of the Mesh-like protein in *C. maenas* (accession no. GBXE01116262.1), consisting of 1308 amino acids. Green dots indicating N-glycosylation motifs. (Protter–version 1.0).

## 3.2 Relative transcript abundance of individual Septate Junction proteins in the anterior gills of different haline crab species: *Carcinus maenas*, *Metacarcinus magister*, and *Geothelphusa dehaani*

In order to obtain an overview of transcript abundance and possible importance of the particular Septate Junction proteins in the gill epithelia of the euryhaline green shore crab *C. maenas* (natural salinity range 4 - 32 ppt), the marine osmoconformer *M. magister*, and the true freshwater Japanese crab *G. dehaani*, branchial expression levels relative to *mega-like* (set to 1) were investigated. For the comparison of mRNA abundance of the various SJPs the anterior gills of the 3 different haline crabs were chosen, as the epithelia of the osmoregulatory posterior gills consist of a mix of mitochondria-rich cells and mitochondria-poor pavement cells. The anterior gills of all crabs consist mostly of simple, mitochondria-poor pavement cells (Freire et al., 2008). Making inter- and intra-species comparisons more relevant.

### *3.2.1 Relative mRNA expression of Septate Junction proteins in anterior gills of Carcinus maenas*

In *C. maenas*, regardless of salinity acclimation transcript abundance patterns were similar. Under both osmotic conditions, expression of the *mega-like* and *kune-like* were highest, with slightly increased in mRNA abundance of *kune-like* in the seawater acclimated crabs ($p=0.07$). Approximately a third of the mRNA expression strength compared to *mega-like* was detected for *wrm1-like*. In seawater acclimated crabs, relative mRNA expression of *nrx-IV-like*, *gli-like*, and *cont-like* was about 7-9 times lower when compared to *wrm-1-like*. In brackish water acclimated crabs the relative mRNA expression of *nrx-IV-like* and *gli-like* was ca. 9 times lower expressed when compared to *wrm1-like*, but in contrast expression levels of *cont-like* was ca. 33 times lower when compared to *wrm1-like.* Of all genes *mesh-like* displayed the lowest expression levels in *C. maenas*. In seawater acclimated crabs it was ca. 270 times lower expressed than *wurm1-like*, in brackish water acclimated crabs ca. 68 times lower expressed than *wurm1-like* (Fig. 25 A, B).

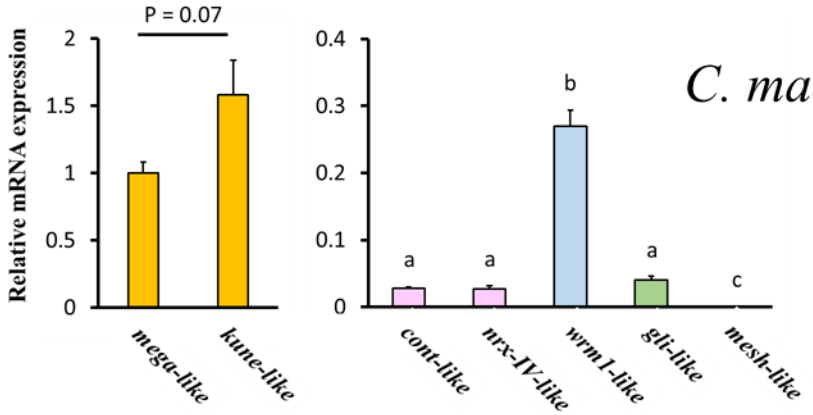
### *3.2.2 Relative mRNA expression of Septate Junction proteins in anterior gills of Metacarcinus magister*

In contrast to the observed relative expression pattern of SJPs in *C. maenas*, in the marine osmoconformer *M. magiste*r relative expression of the *wrm1-like* was highest. Followed by *cont-like* and *kune-like* with a ca. 2.3 and 1.9 times lower mRNA abundance in the gills, respectively, compared to *wrm1-like*. A lower mRNA expression was observed for *nrx-IV-like* and *mega-like* with respective ca. 3.4 and 4 times lower mRNA abundance in the gills when compared to *wrm1-like.* Relative abundance of *gli-like* was lowest, ca. 9.3 times lower when compared to *wrm1-like.* Note due to the lack of high efficiency qPCR primers, relative expression of *mesh-like* could not have been conducted (Fig. 25 C).

### *3.2.3 Relative mRNA expression of Septate Junction proteins in anterior gills of Geothelphusa dehaani*

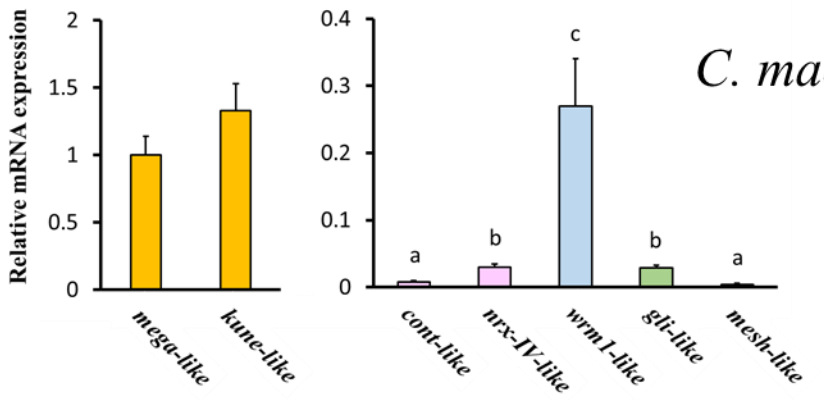
In the freshwater Japanese crab *G. dehaani* relative mRNA expression of *kune-like* was highest in the anterior gill followed by *cont-like* and *mega-like* which showed a ca. 2.6 and 4.9 times lower relative mRNA expression when compared to *kune-like*. Low relative mRNA expression levels were detected for *nrx-IV-like*, *wrm1-like* and *gli-like*, displaying ca. 35, 33 and 44.5 times lower branchial expression levels when compared to *kune-like*, respectively (Fig. 25 D).

A



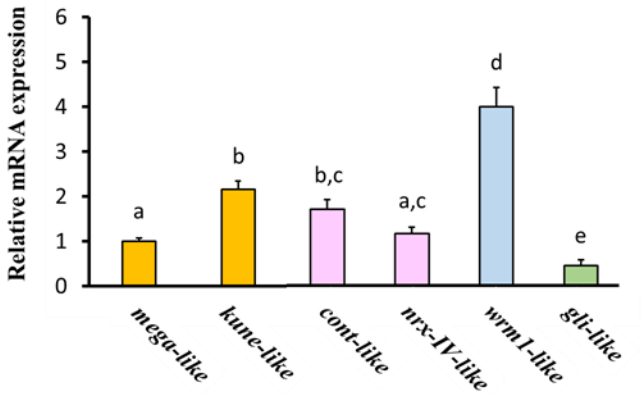

*C. maenas* (SW)

B



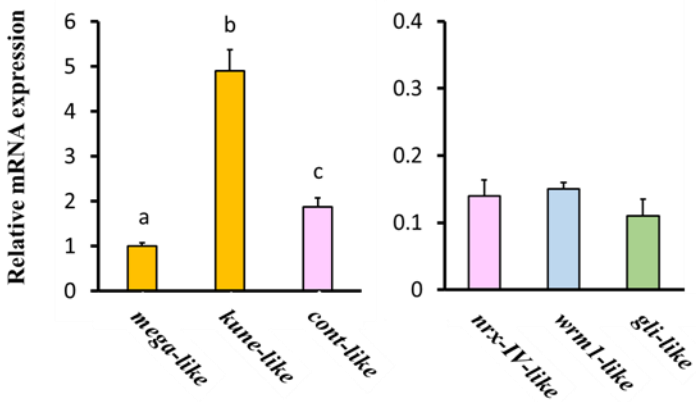

*C. maenas* (BW)

C




*M. magister* (SW)

D




*G. dehaani* (FW)

**Figure 25:** Relative mRNA expression of SJ-like proteins in anterior gills of *C. maenas*, *M. magister*, and *G. dehaani*. For seawater (n = 5) (A) and brackish water (n = 5-7) (B) acclimated *C. maenas* mRNA was expression is shown relative to the ribosomal protein S3. C) For marine *M. magister* mRNA was measured relative to the α-subunit of the $Na^+/K^+$ -ATPase (n = 4 - 5). D) Due to the lack of gill tissue and corresponding RNA, the RNA expression analysis (n = 5 - 6) was conducted using NORMA-gene method (Heckmann et al., 2011). The relative expression of *mega-like* was set to 1. Shown are means ± SEM. Different letters display statistically significant differences between genes. For all, a one-way ANOVA with Game-Howell post hoc test pairwise comparisons were conducted. For *C. maenas* differences between *mega-like* and *kune-like* were analyzed employing an unpaired student's T-test. Statistical difference was assumed with $p < 0.05$).

## 3.3 Tissue distribution of Septate Junction proteins in osmoregulating green shore crabs, *Carcinus maeans*

In order to further characterize the identified potential SJPs found in decapod crustacea, a mRNA tissue expression analysis was conducted employing the osmoregulatory active mitochondria-rich posterior gills (PG), the mitochondria-poor anterior gills (AG), the kidney-like antennal gland (AntGl), the hindgut (HG), and claw muscle (CM) isolated from brackish water acclimated and osmoregulating *C. maenas*. It must be stated and considered that gills consist, with exception of a few neuronal cells, only of epithelial cells. All other isolated tissues consist, besides epithelial/endothelial cells, mostly of connective tissues (AntGl) and muscle cells (HG, CM).

### *3.3.1 Tissue mRNA expression of mega-like*

Relative expression of the *mega-like* was highest in the gills with approximately half of the abundance fund in gill tissues being detected in AntGl. When compared to PG (set to 1), less than 20% of *mega-like* expression was detected in HG. By far the lowest expression was found for mega-like in CM (approximately2% of PG expression) (Fig. 26 A).

### *3.3.2 Tissue mRNA expression of kune-like*

While the expression pattern for *kune-like* in HG and CM mirrored the pattern found for *mega-like*, it showed in AG a 60% higher relative mRNA expression when compared to PG. When a students' T test was applied, AntGl was in tendency (p = 0.06) lower expressed compared to AG (Fig. 26 B).

### *3.3.3 Tissue mRNA expression of cont-like*

The relative mRNA expression pattern of *cont-like* was very similar when compared to *mega-like*. The gills showed highest, but similar expression strength, while AntGl was slightly lower expressed (76% relative to PG). HG showed for *cont-like* approximately 25% of the expression strength as detected in PG and only approximately 3% of the expression reported for PG was detected in CM (Fig. 26 C).

### *3.3.4 Tissue mRNA expression of nrx-IV-like*

As seen for *kune-like*, also for *nrx-IV-like* relative mRNA expression was highest in AG and approximately 55% higher when compared to the expression level found in PG. In AntGl relative *nrx-IV-like* expression was approximately 70% of the expression found in PG. Also for *nrx-IV-like* low expression levels were detected in HG (approximately 35% of PG) and lowest expression values were found in CM, accounting for just approximately 1% of the expression in PG (Fig. 26 D).

### *3.3.5 Tissue mRNA expression of wrm1-like*

For *wrm1-like* similar relative mRNA expression levels were found in the gills and AntGl. Here, in contrast to all other investigated SJPs, expression was lowest in HG, account only for approximately 1% of the expression levels found in PG. In CM the relative mRNA expression was considerablyincreased, when compared to other SJPs and were measured to be 10% of the expression strength found in PG (Fig. 26 E).

### *3.3.6 Tissue mRNA expression of gli-like*

As for *wrm1-like*, also relative mRNA expression levels in PG, AG, and AntGl were similar for *gli-like*. Relative expression of *gli-like* in HG was low, accounting for ca. 15% of the expression strength found in PG, but again, lowest in CM (ca. 3% of PG) (Fig. 26 F).

*3.3.7 Tissue mRNA expression of mesh-like*

As for *gli-like* and *wrm1-like* also for the relative mRNA expression of *mesh-like* no differences in expression levels were detected between PG, AG, and AntGl. Expression of mesh-like in HG was relatively high and measured to approximately 37% of the expression levels found in PG, whereas expression in CM was again lowest, accounting to approximately 8% of the expression in PG (Fig. 26 G).

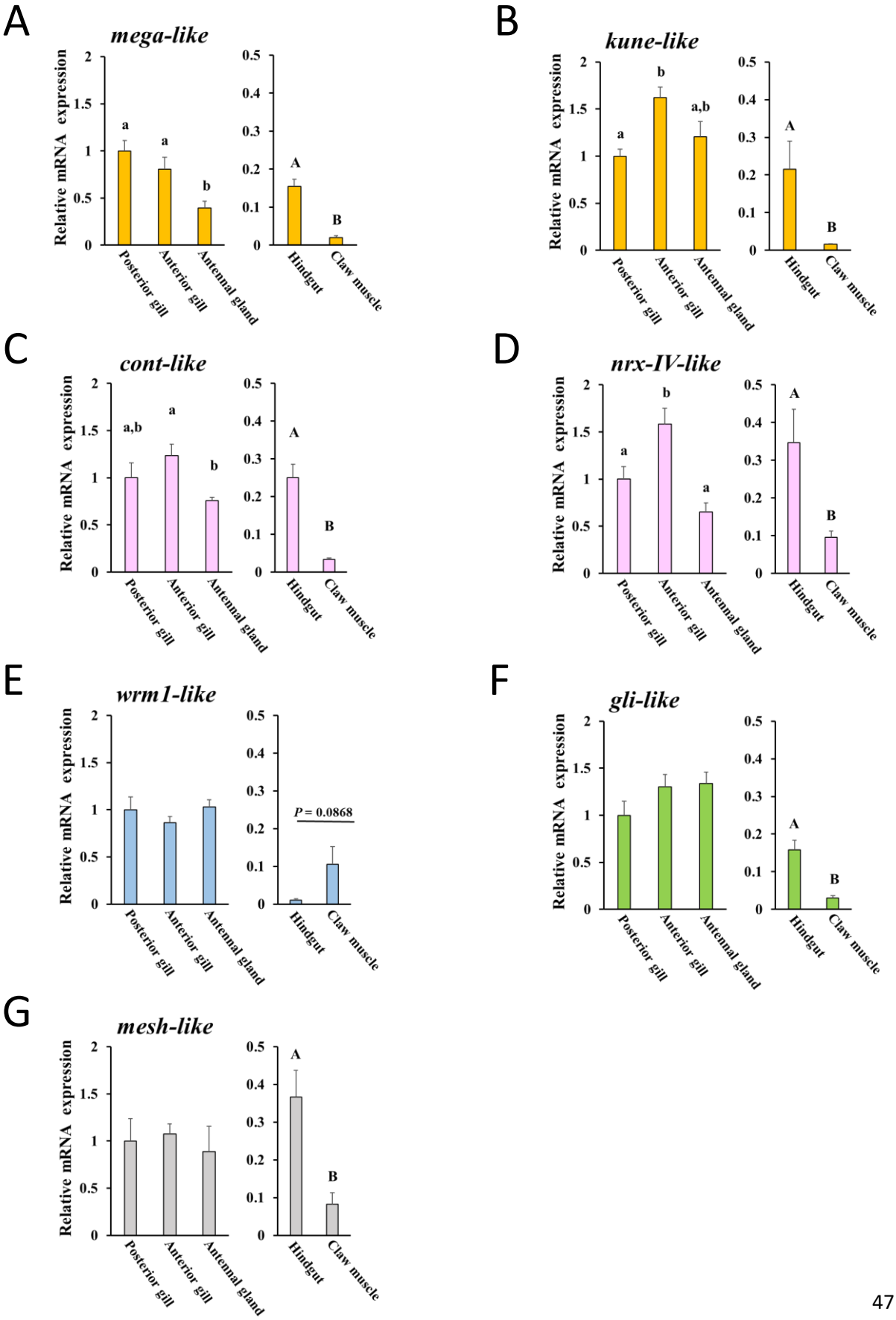

A
mega-like
Relative mRNA expression
a
a
b
A
B
Posterior gill
Anterior gill
Antennal gland
Hindgut
Claw muscle
B
kune-like
a
b
a,b
A
B
C
cont-like
a,b
a
b
A
B
D
nrx-IV-like
a
b
a
A
B
E
wrm1-like
P = 0.0868
F
gli-like
A
B
G
mesh-like
A
B

**Figure 26:** Relative mRNA expression levels of SJPs in various tissues of brackish water acclimated *C. maenas*. A) *mega-like* (N = 5-6); B) *kune-like* (n = 5-6); C) *cont-like* (n = 5-6); D) *nrx-IV-like* (n = 5-6); *wrm1-like* (n = 5-6); *gli-like* (n = 5-6); *mesh-like* (n = 5-6). Messenger RNA levels were normalized using the NORMA-gene method (Heckmann et al., 2011). For all comparisons, relative mRNA expression for *mega-like* was set to 1. Data are expressed as means ± SEM. The tissues anterior gills, posterior gills, and the antennal gland were analyzed employing one-way ANOVA with Game-Howell post hoc pairwise comparisons. For statistical analysis of the hindgut and claw muscle an unpaired student's T-test was employed (capital letters). Different letters indicate significant differences between tissues. Statistical difference was assumed with $p < 0.05$).

## 3.4 Effect of salinity on Septate Junction proteins mRNA expression in osmoregulating posterior gills and the antennal gland in green shore crabs, *Carcinus maeans*

The green shore crab thrives in estuaries (Frederich & Lancaster, 2023), but can also be found in marine and brackish water environments such as the waters of the Baltic sea near the Kieler Bucht, Germany. In brackish water (Salinity = 10 ppt) the posterior gills are predominately responsible for active NaCl uptake to counter passive NaCl losses and to keep hemoplymph osmolality at approximately 700 mOsmol $kg^{-1}$, which is approximately 400 mOsmol $kg^{-1}$ higher than the surrounding water. Due to the inwardly directed osmotic gradient the animals gain water. This water is excreted by the antennal gland, producing a hypotonic urine. In seawater acclimated, osmoconforming green crabs, very little NaCl transport occurs over the gills, while the osmolality of the urine is identical to the osmolality measured in the hemolymph. Consequently, no water is excreted by the antennal gland (Nash et al., 2022).

To evaluate the importance and participation of the identified potential septate junction proteins in branchial and antennal NaCl (and other ions) and water permeability, expression levels of SJPs in both tissues were isolated from osmoconforming and osmoregulating animals.

In the posterior gills all SJPs but *cont-like* and *nrx-IV*-like showed a higher mRNA abundance in branchial tissues from osmoregulating animals. In the antennal gland *mega-like* showed a slight but not significant (P = 0.11) higher relative expression level in osmoconforming animals. No difference in antennal gland mRNA abundance was detected for *kune-like*, *cont-like*, *nrx-IV-like*, and *gli-like*, regardless of the acclimation salinity of the animals. In the antennal gland of osmoregulating crabs both *wrm1-like* and *mesh-like* displayed a approximately 4-times higher RNA expression level when compared to tissues isolated from osmoconforming crabs (Fig. 27).

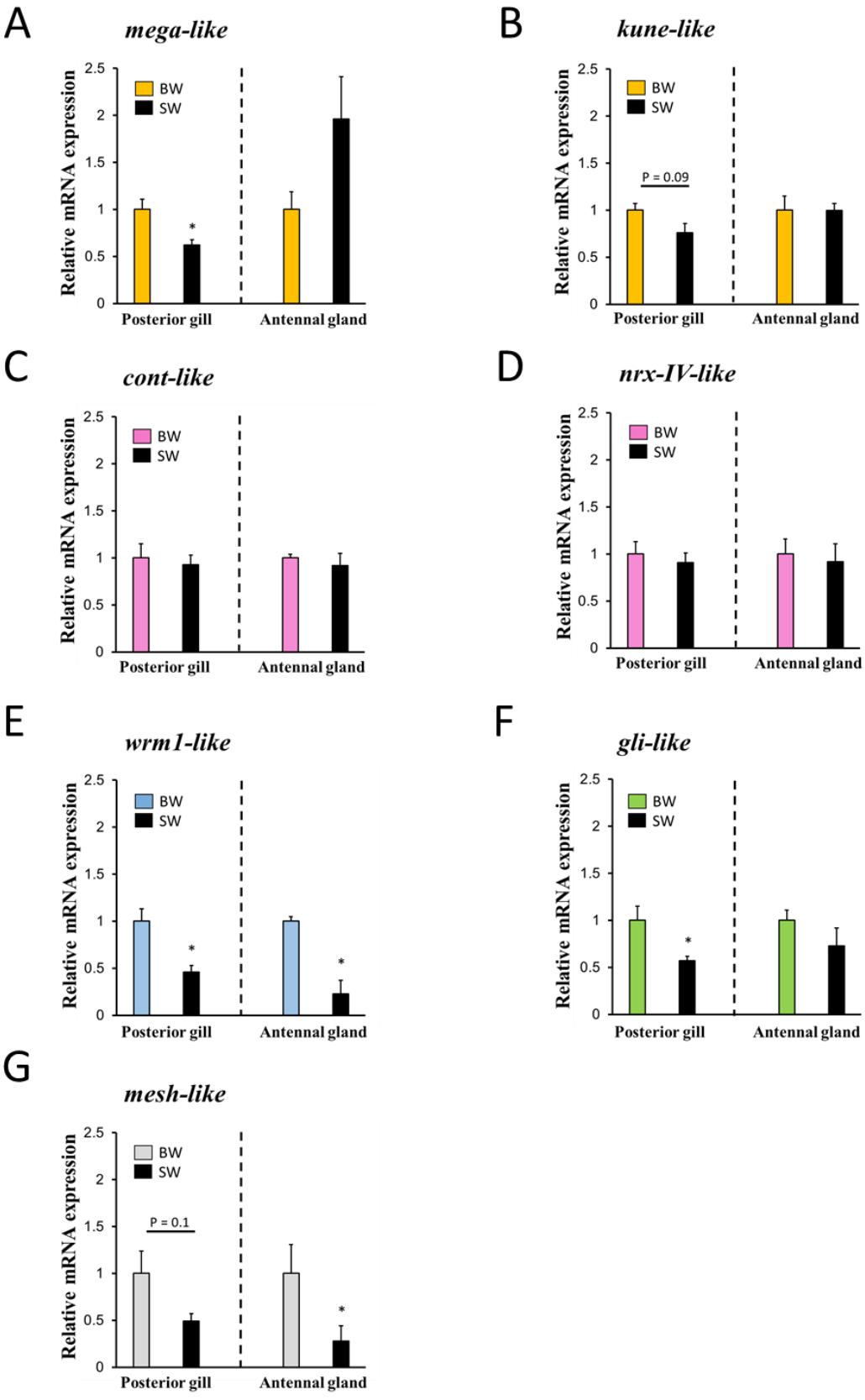

A
mega-like
Relative mRNA expression
BW
SW
*
Posterior gill
Antennal gland
B
kune-like
P = 0.09
C
cont-like
D
nrx-IV-like
E
wrm1-like
F
gli-like
G
mesh-like
P = 0.1

**Figure 27:** Comparison of relative mRNA expression levels of SJPs in posterior gills and the antennal gland from green shore crabs, *C. maenas*, acclimated either to brackish water (10 ppt, colored bar) or full strength seawater (32 ppt, black bar). A) *mega-like* (N = 4-5); B) *kune-like* (n = 4-5); C) *cont-like* (n = 4-6); D) *nrx-IV-like* (n = 5-6); *wrm1-like* (n = 5-6); *gli-like* (n = 5); *mesh-like* (n = 5-6). Messenger RNA levels were normalized using the NORMA-gene method (Heckmann et al., 2011). For gill comparisons, relative mRNA expression for brackish water acclimated animals were set to 1. For comparisons of the antennal gland, also relative mRNA expression for brackish water acclimated animals were set to 1. Data are expressed as means ± SEM. Gill tissue and antennal gland tissue comparisons were analyzed employing an unpaired student's T-test. * indicates significant differences between tissues. Statistical difference was assumed with $p < 0.05$).

## 3.5 Effect of high environmental ammonia levels (HEA) on Septate Junction proteins mRNA expression in posterior gills of osmoregulating green shore crabs, *Carcinus maeans,* acclimated to brackish water

In this series of experiments, the effect of short and long-term (2 and 7 days) exposure to high environmental ammonia (HEA, 1 mM $NH_4Cl$) on branchial expression of SJPs was investigated. As ammonia, a small molecule of ca. 1.45 Å (hydrated $NH_4^+$), is toxic (Larsen et al., 2014) and its branchial passage is likely regulated by SJPs, the aim was to identify SJPs that may respond to this particular environmental stress and may play a role in restricting ammonia influxes.

A 2-day exposure to HEA caused a substantial (ca. 10 to 45-fold) increase of mRNA expression in all investigated SJPs relative to the controls (set to 1), with the exception of mesh-like (Fig. 29; light grey bars). After 7 days of HEA exposure *mega-like, kune-like, cont-like, nrx-IV-like,* and *gli-like* resumed to control levels, while for *wrm1-like* a slight but significant down-regulation was observed. As the only gene, transcripts of *mesh-like* were more than 2-fold up-regulated (Fig. 28, dark grey bars).

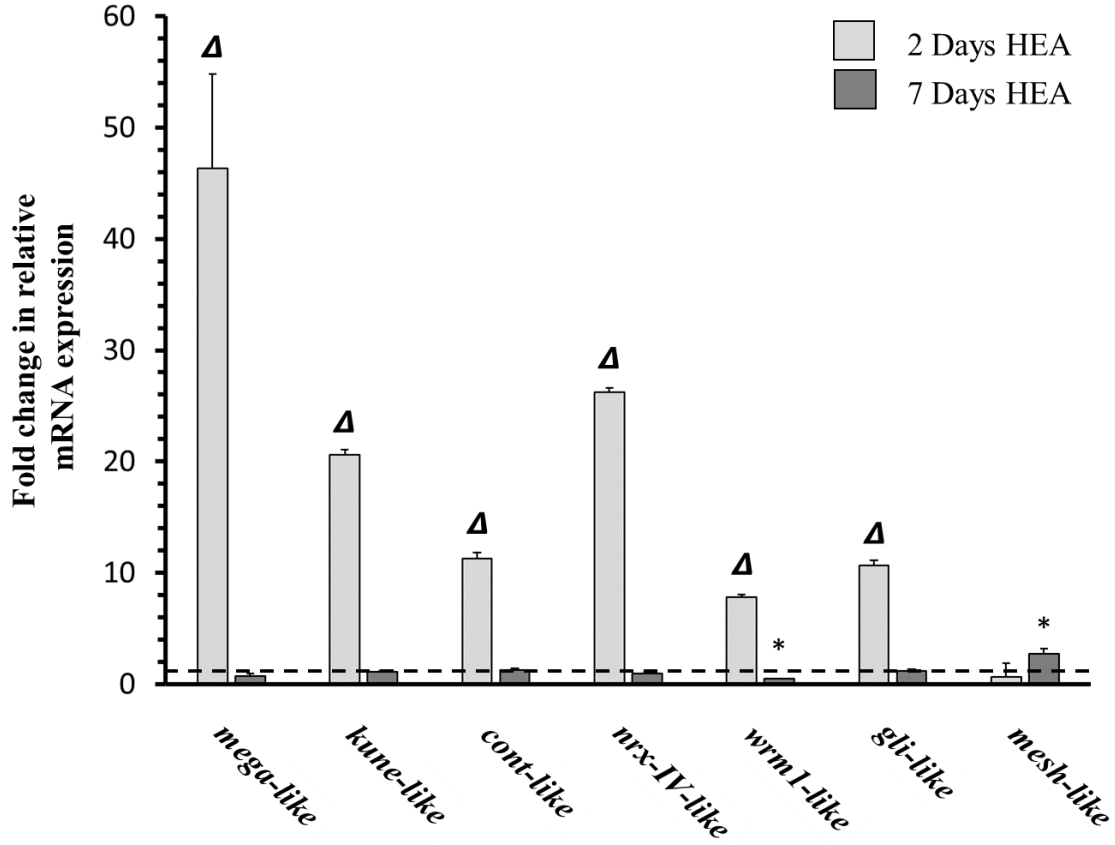



**Figure 28:** The effect of short-term (2 d) and long-term (7 d) exposure to high environmental ammonia (HEA, 1 mM $NH_4Cl$) on mRNA expression of putative SJPs in posterior gills of the green crab *C. maeans* acclimated to brackish water. Data are expressed as means ± SEM (n = 4-7). **Δ** indicates significant difference to control values after a 2-day HEA exposure (unpaired student's T-test); * indicates significant difference to control values (set to 1, dotted line) after a 7-day HEA exposure (unpaired student's T-test).

# 4.0 Discussion

Before discussing the findings of this thesis, it must be mentioned that, besides of a handful of studies on insect SJPs and one investigation on cnidarians, to the best knowledge of the author nothing has been published with regard to proteins being part of the epithelial Septate Junction protein complex in the animal sub-kingdom of invertebrates. Also, although insects and crustaceans are grouped together in the clade of Pancrustacea, the phylogenetic distance is considerable as it is predicted the groups diverged in the Cambrian period, some 520 million years ago (Giribet & Edgecombe, 2019).

## 4.1 Identification of Septate Junction proteins in transcriptome of decapod crabs

### *4.1.1 Presence and sequence analysis*

With regards to amino acid similarities to their insect counterparts (37-54% identity and 52-72% similarities in the amino acid (AA) sequences, respectively) as well as the predicted secondary structures, it can be assumed that the crustacean genes identified in this study expressed in the crab tissues are at least insect-SJP-like genes. However, there is one notable exception: the sequence that was initially considered (and named in the results accordingly) *wrm1-like*. Although the sequence had a 43% identity to the AA sequence of the insect counterpart and contained only one predicted transmembrane domain (TMD), the predicted open reading frame was 85 AA shorter, when compared to the insect Wrm1 proteins (157-162 AAs). This may be a clear indication that the identified crustacean sequence had a different function. In addition, also the predicted extracellular portion of the crab protein (AAs 1- 26) showed a low degree of conservation with only 3 identical AAs including the initial methionine with respect to the insect Wrm1 protein. Therefore, we chose to name the protein Würmchen1-like.

It could further be confirmed that Mega-like and Kune-like proteins are most likely claudin-like proteins, as already suggested for their insect counterparts (Jonusaite et al., 2016b),. As for the vertebrate claudins, their sequences contain 4 trans membrane domains, with a cytoplasmatic N-terminus and C-terminus. In the first extracellular loop of all vertebrate claudins as well as the Mega and Kune proteins found in insects and Mega-like and Kune-like proteins discovered in crabs, a motif (GLW-X-X-C) was detected. This motif seems to be extremely well-conserved as it also can be found in other molluscs (e.g. *Biomphalaria pfeifferi*, accession no. GFMW01028932.1 and GFMW01101741.1, respectively) and planaria (e.g. *Schmidtea*

*mediterranea* accession no. GBEE01007409.1 and GFPN01106374.1, respectively). Unfortunately, the specific function of this conserved motif in vertebrate claudins or invertebrate claudin-like proteins is not known (Anderson & Van Itallie, 2009; Günzel& Yu, 2013; Nelson et al., 2010). Note, as shown in vertebrate claudins, insect and crab claudin-like proteins tryptophan is located upstream of the motif and cysteine is found downstream of the motif (still in the first extracellular loop). These amino acids are still considered as part of the motif, but the location of the amino acids within the sequence is variable.

*4.1.2 Relative transcript abundance of SJ- like protein in the anterior gills of different haline crabs*

As seen in Fig. 28, branchial mRNA expression strength of the SJ-like proteins relative to each other was strikingly similar in *C. maenas* regardless whether the animals were acclimated to a salinity where crabs are osmoconforming (acclimated to 32 ppt) or hyper-regulating (acclimated to 10 ppt). This kind of conserved expression pattern within one species can also be observed in gills of the whiteleg shrimp, *Litopenaeus vannamei*, also a decapod crustacean like the true crabs. For this commercially relevant species an initial transcriptome project was conducted exposing the euryhaline shrimps for 30 days to either seawater (salinity = 30 ppt), brackish water (salinity = 15 ppt), or freshwater (N=2) (Zhang et al., 2016). In their expression analysis the authors have not considered potential SJPs, however the data are available at the Crustybase website (https://crustybase.org/) and a tblastn search was conducted in this study.

As seen in Fig. 29, also for this shrimp a very similar expression pattern was observed regardless of the acclimation salinity.

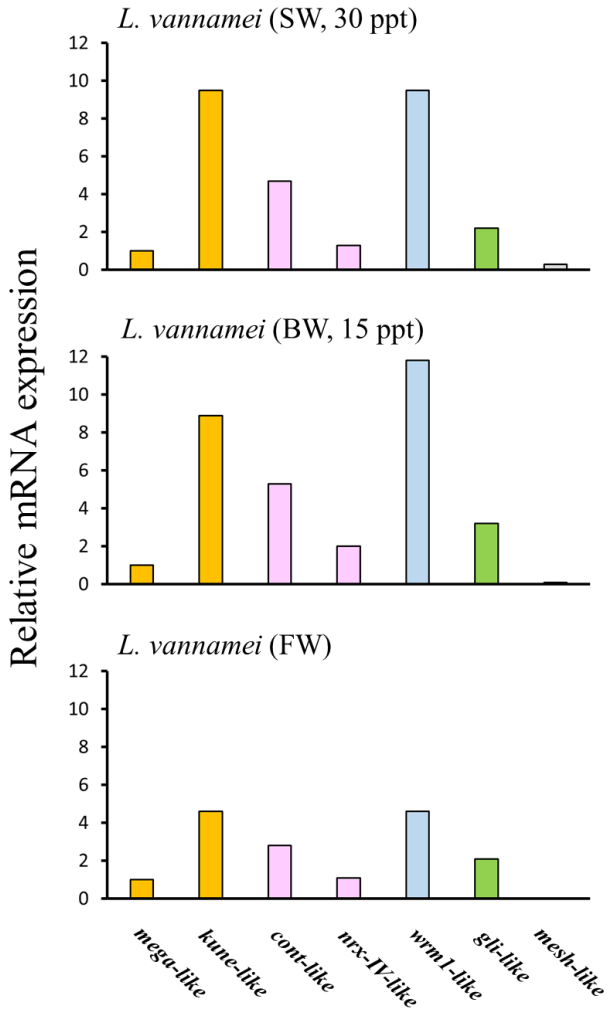



**Figure 29:** Relative mRNA expression levels of SJ-like proteins in the gills of the whiteleg shrimp *L. vannamei* acclimated for 30 days to seawater (30 ppt), brackish water (15 ppt), and freshwater. Data mined from (https://crustybase.org/) (n = 2). In this analysis expression of mega-like was set to 1.

Overall however, the expression pattern from the shrimp differs from the pattern found in *C. maenas*. In the shrimp, claudin-like SJPs , have a much lower transcript abundance relative to *cont-like*. In contrast, similar transcript abundance was observed between *mega-like*, *kune-like* and *cont-like* in the seawater crab. *M. magister*. For all investigated crustaceans *mega-like* tended to be or was significantly lower in transcript abundance, compared to *kune-like*. Further, comparing the expression pattern of the SJP-group *nrx-IV-like*, *wrm1-like*, and *gli-like* in *C. maenas*, *M. magister*, and the shrimp *L. vannamei*, it can be stated that *wrm1-like* displayed a more pronounced expression (*ca*. 4 - 6 times higher expression levels) when compared to other transcripts from this group. In the true freshwater crab *G. dehaani*, *wrm1*-like was not different from *nrx-IV-like* and *gli-like*. In *C. maenas* and also *L. vannamei* (no data available for *M. magister* and *G. dehaani*), *mesh-like* showed by far the lowest expression.

Mesh shows a relatively high conservation between insects and crabs with 51% and 65% identity and similarity, respectively, in the respective amino acid sequences. In knock-down experiments employing adult *Drosophila* flies, it was shown that in insects Mesh is important in maintaining the transepithelial potential difference ($PD_{te}$) as well as the transepithelial fluid and ion transport (Jonusaite et al., 2020). This hints to an important barrier function of Mesh for major ions ($Na^+$, $K^+$, $Cl^-$), known to play a role in generating an actual detectable $PD_{te}$ and are used to drive transepithelial fluid, ion, and nutrient transports in insects (Jonusaite et al., 2020). It is therefore remarkable that only a very low mRNA expression can be found in the gills of osmoregulating *C. maenas* and *L. vannamei*.

Overall, considering the sparse data available for crustaceans, it can be observed that from the 2 identified claudin-like proteins expressed in gills, *kune-like* displays a higher transcript abundance when compared to *mega-like*. In addition, an elevated relative abundance of *wrm1-like* seems to be required. Note, in *Drosophila*, Würmchen1 is a core component of the SJP-complex. It has been shown to be required for the organization of the SJP-complex during embryogenesis and is essential for the barrier function of the paracellular pathway (Königsmann et al. 2020). At the same time, we must not assume that Wrm1-like in crustaceans has the same function as described for insects due to its considerable dissimilarities in protein composition and length.

Particularly, with the discovery that very similar SJP expression patterns were found within one crustacean species when acclimated to various salinities, it seems safe to say that each species has its own, specific composition of SJ-like proteins, with a species-specific abundance pattern of the particular SJ-like proteins. As analyzed in the result section, SJ-like proteins investigated in this study are fairly conserved among the crabs investigated, displaying here $\geq$ 81-90 % identity in their respective amino acids sequence and an even higher percentage of AA similarities, suggesting for all a similar function within the SJ-complex.

It is to assume that due to evolutionary processes SJP complex compositions are optimized for the particular needs and life-styles of an animal. In contrast to the marine osmoconforming Dungeness crab, *M. magister* and the stenohaline Japanese freshwater crab *G. dehaani*, the green shore crab, *C. maenas*, often lives in shallow coastal areas and estuaries and can frequently be found during low tide on land (hence the common name “shore crab) where they are often exposed to air (Frederich & Lancaster, 2023). Due to the tidal rhythm, the crabs experience frequent and

regular changes of salinities and osmotic pressures, but also a fairly rapid change of environmental solutes, including $Na^+$, $K^+$, $Mg^{2+}$, $Ca^{2+}$, and $Cl^-$ when in estuaries, while keeping hemolymph concentrations of these ions constant (Winkler et al., 1988). Consequently, these crabs must be "prepared" to respond to rapid changes of the environment in order to maintain homeostasis. It is conceivable that also the branchial SJP complex and its protein expression pattern are laid out in a way to cope with these environmental changes with little adjustments, e.g. massive structural reorganization and gene up and down-regulations to save energy. In support of this assumption is the fact that the whiteleg shrimp does not live in a constant environment. During their juvenile, adolescent, and sub-adult stages the animals are found in coastal estuaries, lagoons or mangrove, all areas with varying salinities. As seen for *C. maenas*, also *L. vannamei* displays a very similar SJP expression pattern, regardless of their acclimation salinity.

### 4.2 Crab SJ-like proteins found in epithelia/epithelial-rich tissues

In order to further characterize the crustacean SJ-like proteins identified in the crabs, an mRNA expression analysis in various tissues of the hyper-regulating green shore crab was conducted. As guardians of the paracellular pathway, SJPs should be expressed, more or less exclusively in epithelia as shown for insect tissues (Jonusaite et al., 2016b). And indeed, as seen in Fig. 27, all 7 investigated crab SJPs showed highest tissue expression in the gills, a tissue that is predominantly composed of epithelial cells (Venezia et al., 1992; Compere et al., 1989; Freire et al., 2008). In hyper-regulating *C. maenas*, only the posterior gills develop when acclimated to low salinity, patches of mitochondria-rich cells (Compere et al., 1989). The posterior gills are known to be mostly responsible for active NaCl uptake, indicated by a higher abundance and activity of the $Na^+/K^+$-ATPase (Siebers et al., 1982) and a higher transepithelial potential compared to anterior gills. Due to these described ion transport processes and the resulting electrochemical gradients, and a lower transepithelial conductance in the posterior gills (41 mS $cm^{-2}$) when compared to the anterior gills (62 mS $cm^{-2}$), it was assumed that the posterior gills exhibit higher expression levels of SJPs. This however, was not the case, in fact, higher transcript abundance of *kune-like* and *nrx-IV-like* was observed in anterior gills of the green shore crab . It must be noted that in brackish water acclimated green crabs, the posterior gills are considerably larger (larger volume) (Fig. 31), with more mitochondria and with deep and elaborated membrane

infoldings and thereby likely also more membrane proteins. Additionally, it was shown that key enzymes and transporters related to the osmoregulatory NaCl uptake, such as $Na^+/K^+$-ATPase, $Na^+/H^+$ exchanger, $HCO_3^-/Cl^-$ anion exchanger, $Na^+/HCO_3^-$-co-transporter, carbonic anhydrase 1 and 2, display a significant higher mRNA abundance in the posterior gills (Fehsenfeld et al., 2013). The SJP-complex, by contrast, comprises likely only a small sealing band around the cells. The author speculates, that the SJP mRNA: total RNA ratio, is thereby much smaller in the posterior gills as when compared to the anterior gills. Accordingly, as for the cDNA synthesis total RNA was employed, mRNA expression values for the SJP in the posterior gills are most likely underestimated.

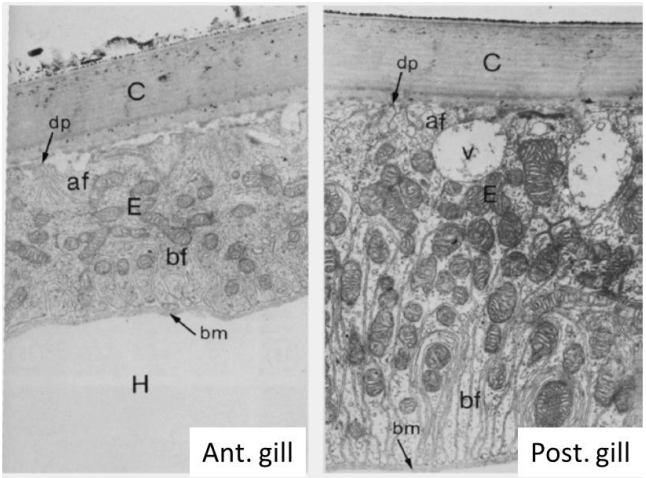



**Figure 30:** Cross sections of anterior (16,000 x magnified) and posterior gills (12,000 x magnified) from brackish water acclimated *C. maenas* localized near the afferent vessel. af, apical membrane infoldings; bf, basolateral membrane infoldings; bm, basal lamella; C, cuticle; E, gill epithelium cell; dp, dense plaque (modified after Compere et al. 1989).

When compared to branchial expression levels, similar high mRNA abundances were observed in the kidney-like antennal gland for all SJPs. The antennal gland is the primary excretory organ in decapod crabs. In principle it resemples the organization of a single nephron in the vertebrate kidney (Freire et al., 2008). This multifunctional organ is involved in eliminating excess extracellular water, reabsorbing ions and small organic molecules (e.g. amino acids, glucose) from the previously ultrafiltrated primary urine, and maintaining extracellular acid-base status (Henry & Weihrauch, 2023; Allen et al., 2021). Although not quantified, sections of the antennal gland from another osmoregulating crab, *Ocypode stimpsoni*, imply that this tissue is very rich in epithelial cells (Tsai & Lin, 2014) but also connective tissues. This suggests that the recorded mRNA abundance is underestimated, but likely only to a minor degree.

This is different to the mRNA expression levels found in the hindgut. Here expression was in general lower when compared to the gills and the antennal gland. This low expression was not expected as the hindgut's function is believed to form feces, including water and ion uptake (Castejón et al, 2021, McGaw, 2013), but may be explained by the way the tissue was prepared. Due to the fragility of the tissue, total RNA was not isolated from epithelial scrapings, but from the whole hindgut. As seen in Fig31 which displays a hindgut of a typical marine crab, the spider crab *Maja brachydactyla*, the epithelia cell layer resembles only a small portion of all the cell types the tissue is composed of, including connective tissues, longitudinal, and circular muscles. In other words, SJP expression levels reported in this study may be considerably underestimated. Notably, *wurm1-like* displayed an extraordinary low mRNA expression in the hindgut, which cannot be explained by a dilution of this specific gene due to other cell types/tissues in the preparation alone and one is tempted to assume that *wurm1-like* plays only a minor role in the hindgut.

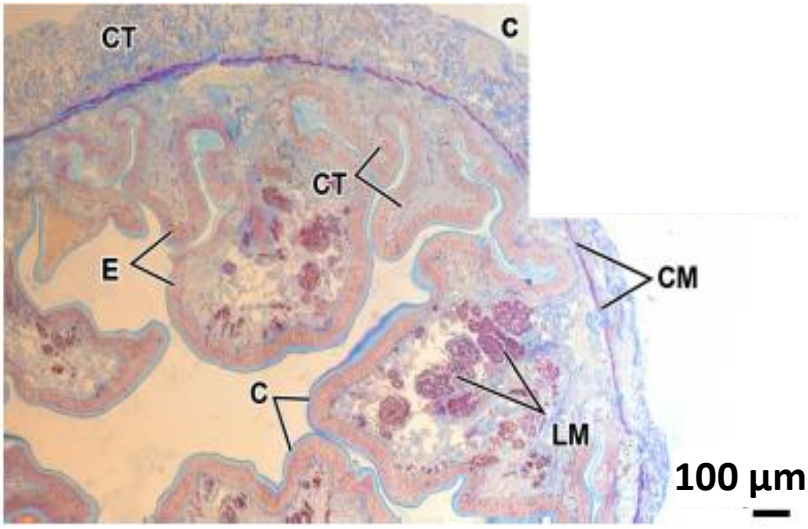



**Figure 31:** Transversal section of the hindgut of adult spider crab *Maja brachydactyla*. C, cuticle; CM, outer circular muscle; CT, connective-like tissue; E, epithelium; LM, inner longitudinal muscles (modified after Castejón et al., 2021).

With the exception of *wrm1-like*, the SJPs investigated in this study are lowest expressed in the muscle tissue. As tight junction molecules in vertebrates can also be found in endothelia, e.g. claudins and occludins (Gonzalez-Mariscal et al., 2003, it is conceivable that Wrm1, but possibly also other SJPs, are localized in the hemolymph vessels supplying the muscle cells with nutrients and oxygen (McGaw & Whiteley, 2023). This would/could explain a SJP presence, however, with low abundance in the muscle tissue.

All together the results show a high abundance of mRNA coding for SJ-like proteins in epithelia, providing additional evidence that the sequences identified in the crabs based on tblastn searches using verified insect SJP proteins are of epithelial/endothelial origin, and thereby likely also part of an SJP-complex.

## 4.3 The effect of salinity changes on branchial and antennal gland expressed SJPs in the green shore crab *Carcinus maenas*

### *4.3.1 The effect of salinity changes on branchial expressed SJPs in the green shore crab Carcinus maenas*

Gills of aquatic organisms are in general in direct contact with their environment and play a vital role maintaining homeostasis of the body fluids of the respective animal. Gills of decapod crustaceans are multifunctional organs and responsible for, e.g. osmoregulatory NaCl transport, $O_2$ and $CO_2$ transport, acid-base balance, nitrogen excretion, and transport of divalent ions. When acclimated to brackish water with a salinity of 10 ppt., *C. maenas* hyper-regulates, maintaining the osmolality of the hemolymph at approximately 700 mOsmol $kg^{-1}$ and thereby about 400 mOsmol $kg^{-1}$ above the osmolality of the environment (Nash et al., 2022). Mainly responsible to this osmoregulatory NaCl uptake are the posterior gills, investigated in this series of experiments.
The gills promote an active uptake of $Cl^-$, which depends on the activity of the $Na^+/K^+$-ATPase and carbonic anhydrase. The activity of these enzymes drive an apical $Na^+/K^+/2Cl^-$ cotransporter and $HCO_3^-/Cl^-$ exchanger. Sodium ions follow *via* the paracellular pathway driven by the generated transepithelial potential difference (Henry & Weihrauch, 2023). The resulting osmotic gradient then drives water into the body fluids, likely through the paracellular pathway. Excessive water is then eliminated *via* the antennal glands, which produces a hypotonic urine (Nash et al., 2022). This requires the active reabsorption of NaCl and other ions and valuable molecules such as amino acids and glucose from the initially ultra-filtrated primary urine, while water must stay in the nephron regardless of the osmotic gradient. Seawater dwelling green crabs are osmoconformers and gills produce only a moderate potential difference ($PD_{te}$) (Siebers et al. 1985). In these crabs the osmolality of the hemolymph and the urine are identical and similar to the osmolality of the surrounding water (Nash et al., 2022). It is important to note that seawater

contains relative to the hemolymph, high concentrations of, e.g., $Mg^{2+}$ (Winkler et al., 1988), ions that should not leak uncontrolled into the hemolymph *via* the paracellular pathway, but need to be excreted *via* the antennal gland.

In the posterior gills of *C. maenas mega-like*, *wrm1-like*, *gli-like*, and showed a moderate higher mRNA expression in animals acclimated to brackish water, while *kune-like* ($p = 0.09$) and *mesh-like* ($p = 0.1$) indicated a tendency of higher mRNA expression levels in animals acclimated to brackish water. No change in mRNA abundance was observed for branchial expression of *cont-like* and *nrx-IV-like* (Fig. 28). This implies that *mega-like*, *kune-like*, *wrm1-like*, *gli-like*, and *mesh-like* are now needed to mitigate the challenges that come with the new environmental conditions, namely outwardly directed gradients for $Na^+$, $Cl^-$, $Ca^{2+}$ (ca. 2-fold), and $K^+$ (ca. 2.4-fold) and inwardly directed gradients for $Mg^{2+}$ (ca. 2.2-fold) and $SO_4^{2-}$ (ca. 2.7-fold). In addition, the now inwardly directed osmotic gradient drives water through the epithelia from the environment into the hemolymph (calculated from hemolymph measurements (Fehsenfeld & Weihrauch, 2013) and the composition of the applied artificial sea salt mix). Although not measured, it can be assumed that the outwardly directed gradient of, e.g., glucose remains rather unchanged. It should be noted that in the euryhaline giant freshwater prawn, *Macrobrachium rosenbergii*, the concentration of free amino acids in the hemolymph increases with increasing environmental salinity, basically doubling when transferred from freshwater to full strength seawater. Free circulating amino acids are likely needed for cellular volume regulation osmoconforming invertebrates (Yang et al., 2001). Regardless, since transcript levels of none of the investigated SJ-like proteins showed an increase, it could be assumed that they are involved in retention of solutes, restricting paracellular loss.

The mRNA upregulation of some SJ-like proteins in the osmoregulating posterior gill are likely part of a now more elaborated SJP-complex. In a study of the euryhaline rainbow crab *Chasmagnathus granulatus* acclimated to various salinities it was shown that the length of the SJP-complex doubled from ca. 6 µm in posterior gills from animals acclimated to 34 ppt to 11.7 µm in gill acclimated to 12 ppt, suggesting a higher overall protein and thereby also mRNA abundance for SJPs (Luquet et al., 2002).

The previously mentioned "pre-adaptation" of the SJPs in the gills to dilute SW can also be observed in the whiteleg shrimp. Here acclimation (30 days) from 30 ppt to 15 ppt caused no change in the expression strength for all investigated SJPs, with the exception of mesh-like, which was down-regulated by ca. 50%. Only when animals were acclimated for 30 days to the extreme

environment of freshwater, was an up-regulation of all SJPs observed (Fig. 32). Interestingly, under these extreme conditions branchial *cont-like* and *nrx-IV-like* were also up-regulated.

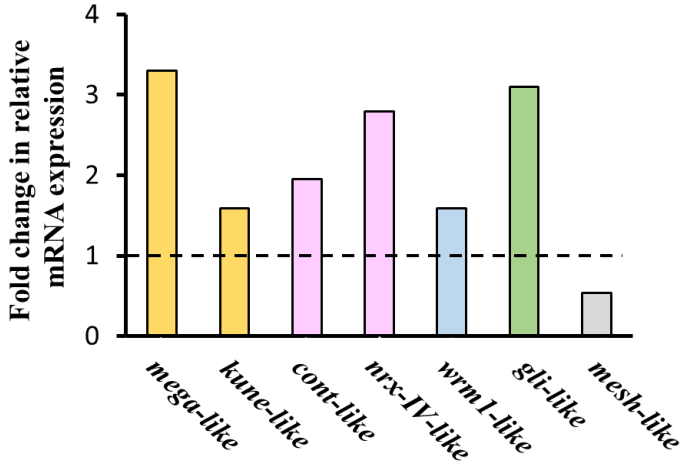



**Figure 32:** Fold change in relative mRNA expression level of SJP, *mega-like*, *kune-like*, *cont-like*, *nrx-like*, *wrm1-like*, *gli-like*, and *mesh-like* in the whiteleg shrimp when acclimated to freshwater. The dotted line represents the respective control value. Data mined from (https://crustybase.org/) (n = 2).

As the only moderately comparable study on SJPs from insects, an investigation of 4 SJPs, Mega, Sinuous, Kune and Neurexin-IV in the gill-like anal papillae from *Aedes aegypti* may be considered. Here fresh-water living mosquito larvae were acclimated to brackish water conditions (Jonusaite et al., 2016a). Similar to the findings in the green crab, when mosquitoes were exposed to brackish water, a condition when NaCl and osmotic gradients between hemolymph and environment are reduced in this usually freshwater dwelling mosquito larvae, mRNA expression of Mega were lower in the anal papillae (gills–like tissue).

### *4.3.2 The effect of salinity changes on SJPs expressed in the antennal gland in the green shore crab Carcinus maenas*

The antennal gland is responsible for maintaining hemolymph osmo-regulation but also to excrete waste-products and divalent cations (Freire et al., 2008). In seawater, *C. maenas* are osmoconformers, no water excretion is required. However, ions, that are passively taken up from the environment, e.g. $Mg^{2+}$, must be excreted, while valuable molecules, e.g., amino acids or glucose (Allen et al., 2021), must be reabsorbed from the primary urine. Similarly, also in brackish water acclimated crabs $Mg^{2+}$ must be excreted (still inwardly directed gradient from the environment) and valuable molecules be reabsorbed, however, now also water must be secreted.

In addition, now also $Na^+$, $Cl^-$, $Ca^{2+}$, and $K^+$ must be reabsorbed from the primary urine under these hyper-regulating conditions. As seen in figure 28B, C, D, and F, mRNA expression levels of *kune-like*, *cont-like*, nrx-IV-like, and *gli-like* remained unchanged, suggesting for these putative SJPs no or only a minor role supporting the antennal gland's function to excrete water and excess ions from the hemolymph. Messenger RNA coding for the potential SJPs Wrm1-like and Mesh-like were more highly expressed in the antennal gland in brackish water acclimated *C. maenas*, suggesting a role in the active reabsorption mechanism of $Na^+$, $Cl^-$, $Ca^{2+}$, and $K^+$ and the excretion of water.

In contrast to the other investigated transcripts, for *mega-like* a higher mRNA expression was detected in the antennal gland of seawater acclimated animals.

As described above, Mega-like is predicted to be a claudin-like protein. In mammals a number of claudins have been characterized for their function in the nephron. Claudin-14, can be found in the thick ascending limb of Henle's loop and has been shown to block the paracellular transport of $Mg^{2+}$. In seawater the inwardly directed $Mg^{2+}$ gradient is about twice that of brackish water (Fehsenfeld et al., 2013; Mantel & Farmer, 1983), and the need to keep $Mg^{2+}$ in the nephron for excretion is accordingly greater to avoid potential toxic effects of hypermagnesemia as described in mammals (Tosto et al., 2024). Although, this can only be a vague speculation that needs to be verified, Mega-like may also exhibit caudin-14-like characteristics, blocking the passive reabsorption of $Mg^{2+}$ in the antennal gland. In any case, the higher abundance of Mega-like in antennal gland in seawater acclimated animals is most likely related to molecules that must stay in the nephron for final excretion in this environment.

### 4.4 The effect high environmental ammonia on branchial expressed SJPs in the green shore crab *Carcinus maenas*

Ammonia is a toxic waste product from nucleic acid degradation and protein metabolism (Larsen et al., 2014). Among other things, in the crayfish *Pacifastacus leniusculus* and in American lobster *Homarus americanus* acclimated to diluted seawater, elevated environmental ammonia caused a disruption of ionoregulatory function (Harris et al., 2001; Young-Lai et al., 1991). Moreover, when *C. maenas* was exposed to 1 mM of $NH_4Cl$, ion permeability of the body surface increased (Spaargaren, 1990).

The obtained data in the posterior gills of brackish water acclimated *C. maeans* showed with the exception of *mesh-like*, a transient but substantial up-regulation of the investigated SJP.

Due to the severe toxicity of ammonia, increasing SJP may be necessary to protect the animals' hemolymph from uncontrolled passive paracellular influxes and to allow for time to strengthen the branchial ammonia excretion mechanism. Unpublished data in *C. maenas* showed that upon exposure to 1 mM $NH_4Cl$, hemolymph concentrations stabilized to near control values within the first 24 hours of exposure after a transient brief 2-fold increase within the first 2 hours (Fehsenfeld et al., 2023). Moreover, in posterior gills of brackish water acclimated *C. maenas*, relative mRNA expression levels of the V-ATPase (subunit B) increased significantly within the first 6 hours after HEA (1 mM $NH_4Cl$) exposure then decreased to near control levels after 14 days of exposure (Weihrauch pers. communication). The V-ATPase is a crucial pump involved in the branchial ammonia excretion mechanism in the green shore crab (Weihrauch et al., 2002). It is noteworthy that the gills from *C. maenas* acclimated to brackish water are still capable of actively excreting ammonia after 2 weeks of HEA (1 mM $NH_4Cl$) exposure, although at a lower rate when compared to controls (Weihrauch pers. communication). At this moment it is too early to speculate potential reasons for the observed down-regulation of wrm1-like and up-regulation of mesh-like after 7 days of HEA exposure.

## 5.0 Conclusions and future studies

The current study provided the first evidence that the investigated transcripts are most likely coding for epithelial SJPs, with some of them being involved in the regulation of ion fluxes during the crab's acclimation to low salinity. However, most important evidence, namely their cellular membrane localization and specific function in the crustacean's SJ-complex, still needs to be explored.

Specific antibodies will show whether or not these potential SJPs are indeed localized at the SJP-complex. Also, knocking-down particular SJPs, e.g. by RNAi, could provide information regarding their particular role within the SJP-complex. Split gill lamellae can be mounted in micro-Ussing transport chambers, while active transport could be reduced by inhibiting the gills energy metabolism via cyanide or 2,4-dinitrophenol (Goedeke& Shulman, 2021; Nůsková et al., 2010). To measure paracellular fluxes, gradients of particular ion-tracers can be applied, for instance $^{22}Na$, $^{86}Rb$ (for $K^+$), and $^{36}Cl^-$. For a more general approach, the conductance over the split half-lamella can be measured, exploring whether the knock-down of a particular SJP, is responsible to prohibit charged molecules the passage of the paracellular pathway. So far, these type of experiments have not been conducted in any invertebrate SJP.

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# 7.0 Appendices

## Appendix I

Identified Septate Junction proteins in the freshwater Japanese crab *Geothelphusa dehaani* expressed in the tissue of the anterior gills.

### A.1 Megatrachea-like protein

Partial cDNA sequence:

```
CTGTGGGAGTTTTGCTTCAATGGTTTTCGGTATCCAAAATATCAATATGATTACAAGTTTATGGGCTGCAACTATAT
CTTCTCAGAAGAATACAGAATTATATGGTCATGGATGATACCAGCATGGTTTATGGCAGTGCAGACTTTCATGACCA
TTGCCCTGCTGGCCAGCATGACCACACTAGTGACTGTGAGCCTCATCCTAGTGCGATGGCCAATGCAAATGATTATG
CGCTATGAATGGCACTTGACTGGCTTCTGTTTCCTTTGTCAGGCTGTTACAGTTGTGGCAATCTTCTTTGCTGTACT
GGTTTTTGGAGTGATGTGTTGGTCT
```

Translated AA sequence (3rd frame):

```
LWEFCFNGFRYPKYQYDYKFMGCNYIFSEEYRIIWSWMIPAWFMAVQTFMTIALLASMTTLVTVSLILVRWPMQMIM
RYEWHLTGFCFLCQAVTVVAIFFAVLVFGVMCWS
```

### A.2 Kune-kune-like protein

Partial cDNA sequence:

```
TTCATCTCTTGGTTCTTCGGGTTCATCGCAGTCATCGTGTTCGGAGCGATGGGAGACAACAGGGACTGGATGCCGCA
CTGGGAACACAACCACCTCTCCTGGTCCTTCGGTTTAGGTGTGGTAGGAGTTGTGGCCGAGTTCGTGGCGGCAGTAC
TTTACTTGGTGGAGTACCGTATACAGAAGCGCAAGGAATCCTACCGCCAAAGCCACGGCGTCTT
```

Translated AA sequence (1st frame):

```
FISWFFGFIAVIVFGAMGDNRDWMPHWEHNHLSWSFGLGVVGVVAEFVAAVLYLVEYRIQKRKESYRQSHGV
```

### A.3 Contactin-like protein

Partial cDNA sequence:

```
TACACTCAGTATGAAGTCAAGATACAGGCATGGAACAGCCAGGGTCCGGGCCCAATCAGCGAGCCAGTGGAGGTCTT
CACAGCAGAGGACATGCCACAAGTGCAGCCCACAGAGGTGTCTGCCTTTGCCCACAATGCCACCTCCCTCAATGTCA
CCTGGCAGCCCATTGAGCCCACCAGAGAAAACATCCGAGGAAAGCTGGTTGGCTACAGGATAAAGTACTGGCGTCGG
AAGGACAGTGAGACAGACCACGTCATTAAGCTGCAGCGTGGAACAGAACCTCATGGCCTTATTGTGGGGTTGGTGCC
TAACACCTTCTACTGGGTGCGTGTTATGGCATACAACCAGGCAGGCTCTGGA
CCAGAGAGTGAG
```

Translated AA sequence (1st frame):

```
YTQYEVKIQAWNSQGPGPISEPVEVFTAEDMPQVQPTEVSAFAHNATSLNVTWQPIEPTRENIRGKLVGYRIKYWRR
KDSETDHVIKLQRGTEPHGLIVGLVPNTFYWVRVMAYNQAGSGPESE
```

### A.4 Neurexin-IV-like protein

Partial cDNA sequence:

TTCCAGTACGTCTACATCGGCAAGAACGAGTCCATGGCCGAGGGCTTTGTTGGCTGCATCTCGCGGGTCCAGTTTGA
TGACATCTACCCACTCAAGTTCTTTTTCAGCAAGACCGACCGCATGAGATCACAGCAGAGTCAACGTCCTCCATCTT
CGAAGACTACTGCGG

Translated AA sequence (1st frame):

FQYVYIGKNESMAEGFVGCISRVQFDDIYPLKFYFQQDRPHEITAESTSSIFEDYC

### A.5 Würmchen 1-like protein

Partial cDNA sequence:

AGCCAGAATTCTACAACTACACACCCTTCTATGTGGGCATCACCATTTGCACCATCATTGGCATCATTCTGATTATG
GTCAACTGGATCTGCAGCTGCTGCCACGAACACTC

Translated AA sequence (3rd frame):

PEFYNYTPFYVGITICTIIGIILIMVNWICSCCHEH

### A.6 Gliotactin-like protein

Partial cDNA sequence:

CCACGACACGTTCCCAATTGGTACAGCACATGGCAGGCCCTGGACTACCAGAGTGCCTGTCCCCAGAACCTCAAGTA
CATTGGTGCCTCGAATGGCATCAGGAACGGCATCCATGAGGACTGCCTCTACATCAACATCTTCTCACCTTCAGTTT
CCATCACAGTGAATGACATGTACCCAGTGCTCTTCTATGTGCATGGCGGTGACCTGTACCATGGGGCAAGCAACCAG
TTCCCGGGCCACATGCTGGCAGCCTGGGGCCAGGTGGTTGTAGTCACCTTCAACTACCGGCTCGGGGCACTCGGGTT
CCTCAGCACAGGGGATGCCAACTCGCCTGGTAACTATGGCCTCACAGACATGGCAATGGCACTCAAGTGGGTGCACA
CCAACATCCGCTTCTTCAATGGCGATCGCAACAGGATCACCGTCTTCGGGCCAGGAGCAGGTGGGGCTATGGCAGGA
CTGCTTGCTGTCTTGCCACGGACCAAGGATTATGTCAACCAAGTTATTGCCATGAGCGGCTCCCCCCTGGCAGAGTG
GGCTGCACTAGATGATGTGTATCGGGTGCAGAACACATCCCGTGTCTATGGTCTTGAAGTGGGCTGC

Translated aa sequence (3rd frame):

PRHVPNWYSTWQALDYQSACPQNLKYIGASNGIRNGIHEDCLYINIFSPSVSITVNDMYPVLFYVHGGDLYHGASNQ
FPGHMLAAWGQVVVVTFNYRLGALGFLSTGDANSPGNYGLTDMAMALKWVHTNIRFFNGDRNRITVFGPGAGGAMAG
LLAVLPRTKDYVNQVIAMSGSPLAEWAALDDVYRVQNTSRVYGLEVGC

## Appendix II

Identified Septate Junction protein in the Dungeness crab *Metacarcinus magister* in the posterior gills.

### A.1 Mesh-like protein

Partial cDNA sequence:

```
CTTTGGGATTGGACCTTCGACCCTGAAGATGACTTTACGCTGCCCGACGGTAGTCTGGGTCCCGCTGCCGAGGCTAG
CAACATGCTGGTGGTTCACAGAGACTTCGGGATGCATTGGGTGTTGGACGACAAGGAGAAGACAGACGTGGGTAGGT
CaCTCTTCAACCACGACAACTATCGCTCCAGCAACTATTTCTACGACCCTATCTTCGAACCTGAATGGATTATTGAC
CCCGTATTGTCTATAAATGCTACCACGAAGTCTGACGAGATCAAGAGAGTGTGCGGGGACTCCTACCAGTGCCACTT
CGACTACGTAGTCACGCTTCGCAGGGACTTCGCAGACATAACCAAGTATTACCAGGACCAG
```

Translated aa sequence (2nd frame):

```
LWDWTFDPEDDFTLPDGSLGPAAEASNMLVVHRDFGMHWVLDDKEKTDVGRSLFNHDNYRSSNYFYDPIFEPEWIID
PVLSINATTKSDEIKRVCGDSYQCHFDYVVTLRRDFADITKYYQDQ
```