Characterization of the dopamine receptor genes in salmon louse, *Lepeophtheirus Salmonis*.

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List of Abbreviations

Abbreviation	Full name
AA	Arachidonic acid
AC	Adenylyl cyclase
Asp	Aspartic acid
ATP	Adenosine Triphosphate
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CL	Cephalothorax Length
Cys	Cysteine
DA	Dopamine receptor
DPI	Days Post Infection/Incubation
dsRNA	double stranded RNA
ELF	Elongation Factor
Ex	Extracellular loops
GPCRs	G-protein coupled receptors
ISH	*In situ* hybridization
IL	Intracellular loops
L. salmonis	*Lepeophtheirus salmonis*
LsDOP1	*L. salmonis* Dopamine receptor 1
LsDOP2	*L. salmonis* Dopamine receptor 2
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Phe	Phenylalanine
PKA	Protein Kinase A
PLC	Phospholipase C
qRT-PCR	Quantitave Reverse Transcriptase PCR
PCR	Polymerase chain reaction
RACE	Rapid Amplification of cDNA Ends

RNAi	RNA interference
RNPs	Ribonucleic Proteins
RISC	RNA Induced Silencing Complex
RT	Room Temperature
Ser	Serine
SiRNA	Short interfering RNA
TL	Total Length
TM	Transmembrane Domain
TRI reagent	Trizol reagent

Abstract

The Salmon louse is a parasite that has a direct and huge bearing on the economy of the Fisheries industry and survival of wild salmon and trout. It is a major threat for Salmonid population in the Northern Hemisphere, particularly in countries like Norway, Scotland, Ireland and Canada and to the aquaculture industry in Chile. The developing resistances against prevailing prophylactic strategies are increasingly becoming a problem for these nations that heavily depend on fishing and aquaculture for its economy. There is an urgent need to address this issue by developing custom-built strategies to prevent sea-lice infestations.

Dopamine is an important chemical messenger that acts as a neurotransmitter, present in the central nervous system and periphery of both vertebrates and invertebrates. Dopamine receptors have been characterized in arthropods and they are important in regulating sexual function, neuronal development and feeding. In ticks dopamine receptor of type D1 has been shown to be involved in salivary secretions which assist in feeding on the host and dopamine receptor acts over two independent signaling pathways. To explore the role of dopamine receptors in *L. salmonis*, RNA interference studies were carried out. Knockdown of LsDopamine1 was significant but no effect on lice morphology was observed, whereas LsDopamine2 seems to exhibit a changed morphology to some extent. Sequence analysis, structure prediction and phylogeny for two dopamine receptor genes (LsDopamine1 and LsDopamine2) from the salmon louse genome showed that they belong to the family of rhodopsin-like GPCRs, seven-trans membrane spanning domains and show high sequence similarities to the dopamine receptors found in arthropods. Ontogenic expression analysis revealed that LsDopamine1 and 2 are expressed in adult male and copepodids respectively. *In situ* hybridization showed the presence of LsDopamine1 and 2 in subcuticular tissues for copepodids and in tegmental glands type 1 in preadult I female lice.

1. Introduction

1.1 Salmon Louse

The salmon louse (*Lepeophtheirus salmonis* Krøyer, 1838) is an ectoparasite, commonly observed on Atlantic salmon (Salmo salar) in the Northern Hemisphere (Wootten et al., 1982, Pike and Wadsworth, 1999), causing annual losses of several hundreds of millions US dollars worldwide (Johnson et al., 2004). Caligid copepod *L. salmonis* also referred to as sea lice, is a major pathogen of wild and farmed salmon fish (Lees et al., 2008). When attached to the host the salmon louse feeds on blood, epithelial tissues, skin and mucus (Brandal et al., 1976). Sea lice spread occurs during the free-swimming planktonic stages (nauplii and copepodid) as they drift with the water currents and then may infect wild salmonids. (Krkosek et al., 2007, Morton et al., 2004). It is often seen that the intensive salmonid cage-farming is the cause of higher infections on wild salmonid populations (Tully et al., 1999, Bjørn and Finstad, 2002, Morton et al., 2004).

Aquaculture industries of Norway, Scotland, Canada, Chile and Ireland are major producers of salmonids. Salmon louse infestations have a great negative influence on Atlantic salmon farming industry which is also a significant environment problem (Johnson et al., 2004, Boxaspen, 2006, Lees et al., 2008). In addition to the environmental issue, treatment for salmon louse infestations are expensive. The first reported outbreak of *L. salmonis* infestation occurred in 1960's in Norwegian Atlantic salmon farms and similar outbreaks were reported in Scotland in mid-1970's (Pike and Wadsworth, 1999). However, due to an increase in the number of hosts and the high reproductive capacity of the lice, salmon lice have become a major challenge in Atlantic salmon aquaculture industry. There are only a limited range of treatments available against salmon lice such as chemical methods including several therapeutants that can be applied as in-feed additives and bath treatments and biological control using cleaner fish. However, the potential in lice for developing tolerance against available chemotreatments is very high, which increases the need to develop tailor-made treatment methods and tools to control sea lice based on knowledge at its molecular level.

1.2 Biology of *L. salmonis*

Lepeophtheirus salmonis, the salmon louse belongs to the subphylum Crustacea, subclass Copepoda, order Siphonostomatoida, family Caligidae and genus *Lepeophtheirus*. Caligus are natural marine parasites of wild and farmed salmon fishes (Costello, 2006, Hamre et al., 2009, Finstad and Bjørn, 2011) and also infects unrelated fish such as Three-spined stickleback in coastal areas of British Columbia (Jones et al., 2006). The Caligidae family consists of around 559 species in 37 genera and the genus Lepeophtheirus is estimated with about 162 species. L. salmonis is highly specific to salmonids and can parasitize species of salmonids of genera Oncorhynchus, Salmo, Salvelinus and all species of pacific salmon (Kabata, 1979, Pike and Wadsworth, 1999, Boxaspen, 2006, Torrissen et al., 2013).

1.2.1 Life cycle

On wild salmonids, sea lice consist of two genera, *Lepeophtheirus* and Caligus, in which *L. salmonis* is the most common one in Northern Europe. *L. salmonis* was previously reported to comprise of 10 developmental stages in which the chalimus stage was divided into four separate stages (Johnson and Albright, 1991, Schram, 1993). But recently, Hamre et al. (2013) clarified that like most other caligid parasitic copepods, salmon louse has a direct (i.e. a single host) and complex life cycle that is characterised by eight developmental stages (with two chalimus stages instead of four) separated by ecdysis in between. The First two stages of *L. salmonis* are Nauplius I and II, following by one copepodid stage, two chalimus, two pre-adult and the final adult stage (Hamre et al., 2013) (Figure 1.1). The duration of different developmental stages is dependent on the physical environment (Pike and Wadsworth, 1999), such as temperature and salinity are the most important factors. Development from fertilization to adult male and female takes 40 and 50 days at 10 °C respectively (Johnson and Albright, 1991).

The initial larval stages of *L. salmonis* consist of two non-feeding planktonic nauplius stages that survives upon energy reserves within yolk sac (Pike and Wadsworth, 1999, Hamre et al., 2009). Copepodid is the free-swimming infectious planktonic stage where the louse attaches itself to the host via second antenna (Wootten et al., 1982). Following attachment to the host, the copepodid start a parasitic life cycle when it molts to a chalimus stage where the louse physically attaches to the host by a special structure referred to as frontal filament (Pike and Wadsworth, 1999). Second antenna and oral appendages assist the parasite in holding on the fish. Male and female are morphologically distinguishable from the chalimus II stage. Molting after the chalimus II stage gives rise to the first motile pre-adult stage. Two pre-adult stages are followed by fully mature adult female and male which are mobile and found on skin, gills and fins of the host (Johnson and Albright, 1991).

Adult male and female *L. salmonis* vary greatly in size and on Atlantic salmon the adult female takes more time to mature as compared to adult male (Jones et al., 2006, Hamre et al., 2009). Female *L. salmonis* may lose one or both spermatophores and in such cases polyandry is common among them (Todd et al., 2005). The adult female lice can produce eggs per pair of egg strings from 107 to 1220 with 6-11 pairs of egg strings. Moreover, female lice has been reported to survive under laboratory conditions for up to 191 to 2010 days (Heuch et al., 2000; Mustafa et al., 2000).

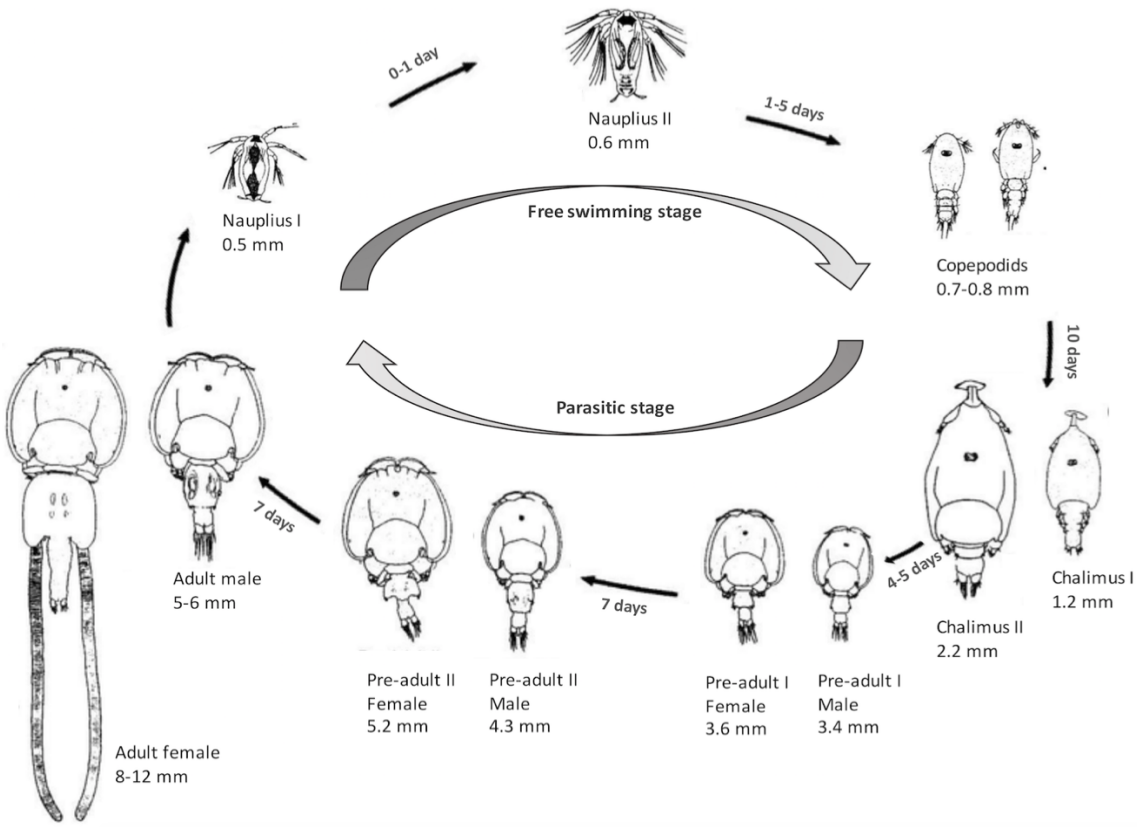


Figure 1.1: Life cycle of Lepeophtheirus salmonis (Krøyer, 1838). Eight developmental life stages of L. salmonis life cycle are represented, each separated by a moult (Hamre et al., 2013). There are three free-swimming planktonic stages, two naupliar stages and one copepodid. Parasitic stages include two chalimus stages followed by two pre adult and the final motile adult stage. The approximate length (in mm) and days between ecdysis are indicated for each life stage in the life cycle. Figure idea is adapted from T.A. Schram, 1993 and modified according to Hamre et al. (2013).

1.3 Infection on salmonids by *Lepeophtheirus salmonis*

Salmon louse infestations can weaken and results in mortality of the host by eating its flesh (Naylor and Burke 2005, Stevenson 2007). This cause physical damage such as skin erosion, bleeding, loss of protective function, tissue damage and deep open wounds at the sites of attachment (Tully and Nolan, 2002). The severity of infection depends the developmental stage of lice (Bjørn and Finstad, 1998), size of the host and on infection density. As a consequence, the pathological impacts on the host includes osmoregulatory failure, reduced growth rate, swimming performance, chronic stress, anemia and the chance of secondary infection can increase (Grimnes and Jakobsen, 1996, Pike and Wadsworth, 1999, Nolan et al., 2000, Bjørn et

al., 2001, Tully and Nolan, 2002, Heuch et al., 2005, Wagner et al., 2008). Infestation by parasitic lice is a significant welfare problem in salmon farming industry. Although salmon lice infections are common to both wild and farmed salmon (Lees et al., 2008). Several initiatives have been taken by the use of available treatments methods to control the serious health issues of salmonids caused by sea lice. Nevertheless, increasingly developing resistance to the currently approved pesticides (Fallang et al., 2004, Espedal et al., 2013) has created an alarming situation.

1.4 Prophylaxis and treatment against *L. salmonis*

In order to avoid costly losses, a number of treatment methods have been developed to prevent and treat sea lice infestations. The biological treatment includes the use of cleaner fish such as ballan wrasse (Labrus bergylta), goldsinny (Ctenolabrus rupestris), crocking (Ctenilabrus melops) and rock cook (Centrolabrus exoletus). Wrasse is a natural predator and feeds on parasites such as sea lice (Treasurer, 2002). The use of cleaner fish has been considered a robust method in treatment for sea lice (Torrissen et al., 2013). It is less expensive, environmentally friendly and non-chemical means of treatment currently being used). Regarding the use of cleaner fish, it is also important to consider the use of clean nets as the cleaner fish also feed on the cage fouling (Treasurer, 2002).

Chemical treatments include several chemotherapeutants that can be used in bath treatment and in-feed treatment. Drugs like organophosphate, synthetic pyrethroids (cypermethrin and deltamethrin) and hydrogen peroxide are used in bath treatments. Organophosphate acts in the nervous system as inhibitors for blocking neurotransmitter acetylcholine esterase (AChE) (Corbett, 1974). Rising resistance against organophosphate detected in the beginning 1990's. As a result, hydrogen peroxide was introduced which chemically breaks down in oxygen and water during treatment and has been shown to be less effective as the lice can survive the treatment (Grant, 2002, Fallang et al., 2004). In addition, oral treatments include emamectin benzoate (Slice), benzoyl ureas, dichlorvos and chitin synthase inhibitor teflubenzuron (Calicide). Reduced sensitivity and increased tolerance due to prolonged use of these drugs have also been documented (Grant, 2002, Espedal et al., 2013, Helgesen and Horsberg, 2013). However, these chemical compounds were not effective for adult parasites.

The Norwegian government has employed a range of management strategies against sea lice infections. These include the reporting of lice numbers, limitations in case of higher numbers of salmon louse on farmed fish and protection of salmon rivers and coastal areas (Serra-Llinares et al., 2014). Moreover, integrated pest management programs have been recommended in several countries. Other control measures employed are good husbandry, fallowing, adequate tidal currents and proper site locations. However, in case of an infection, the treatment of sea lice infestations has been very difficult due to the development of high resistance towards currently available prophylactic regimen that are designed to inhibit the developmental process of sea lice. Study of G protein coupled receptors (GPCRs) in detail can give new insights in the role of dopamine in *L. salmonis*. RNA interference seems to be a promising approach to comprehend the functional relevance of dopamine receptors in salmon louse by targeting its differential expression. This in turn, opens new avenues for designing vaccines and better prophylactic strategies.

1.4.1 Novel Treatments

Characterizing the function of dopamine receptors in salmon louse will facilitate development of novel treatment methods that can be introduced by identifying dopamine receptor antagonists that can prevent the actions stimulated by dopamine. Arthropod dopamine receptors can be explored as novel targets for insecticide development because of their integral roles in neurobiology. Several dopamine antagonists such as clozapine, sulpiride and B-277011A have been used in treatments against many diseases. In ticks, antagonists of dopamine receptor type 1 have been discovered using chemical library screening and comparative pharmacological analyses (Ejendal et al., 2012). GPCRs are extensively targeted for drug development in humans. GPCRs have a high potential for being novel insecticide targets in Arthropods where cases of more than 100 different GPCRs have been identified as targets in genomes of multiple insect species, including malaria and yellow fever transmitting mosquitoes (Hill et al., 2002, Nene et al., 2007). Findings such as these make up the foundation for the rationale of prioritizing functional characterization of GPCRs as potential subjects for insecticide development. Dopamine receptor antagonists have been discovered that have inherent in vivo toxicity towards

mosquitoes (Meyer et al., 2012, Nuss et al., 2015). Thus, dopamine receptors in *L. salmonis* are attractive candidates to explore as new targets for chemical control.

1.5 Dopamine and dopamine receptor

Dopamine is an essential catecholamine neurotransmitter (a chemical that control the flow of information to other nerve cells) in the brain and body, where it is present in relatively high concentrations in the peripheral organs and central nervous system of both vertebrates and invertebrates (Vallone *et al*., 2000, Blenau and Baumann, 2001). Dopamine is involved in a number of functions such as cognition, pleasure, development, memory, learning and modulates neuroendocrine signaling, drug response and motor activity (Missale, 1998, Wilson *et al.,* 1991, Emilien *et al*., 1999, Zhang *et al.,* 2009). Outside the central nervous system, dopamine plays important physiological roles in the periphery as a modulator of cardiovascular function, hormonal regulation, immune system, sympathetic regulation and renal function (Snyder et al., 1970, Iversen and Iversen, 2007). The specific actions of dopamine are mediated via members of G-protein coupled receptor superfamily (GPCRs) (Jaber et al., 1996) and these actions depend on the type of dopamine receptor expressed in the target cell (Sibley and Monsma, 1992, Civelli *et al*., 1993, Jackson and Westlind-Danielsson, 1994, Vallone *et al*., 2000, Beaulieu and Gainetdinov, 2011).

Dopamine receptor (DA) belongs to the family of rhodopsin-like GPCRs, seven-trans membrane spanning domains that are of alpha-helical structures, and consists of extracellular amino terminus, intracellular carboxyl terminus and ligand binding site (Figure 1.2) (Bockaert and Pin, 1999). Dopamine signaling has been center of much research since 30 years because of the involvement of dopamine neurotransmission modifications and misregulation of dopamine signaling as cause of several human disorders such as Parkinson's disease, drug addiction, Tourette's syndrome and Schizophrenia (Vallone *et al.*, 2000, Girault and Greengard, 2004, Kienast and Heinz, 2006, Fuxe *et al.*, 2006).

1.5.1 Types of Dopamine Receptor

In vertebrates and invertebrates, dopamine receptor family has been characterized into two major subfamilies: D1-Like receptors and D2-Like receptors. These receptors belong to the superfamily of GPCRs and possess different functional characteristics, sequence similarity and pharmacological profiles (Missale, 1998, Neve et al., 2004, Pivonello et al., 2007). Evidence of dopamine receptors existence first came in 1972 (Brown and Makman, 1972, Kebabian et al., 1972). D1-Like family receptors are coupled to stimulatory G proteins (G_s) which thereby activate adenylyl cyclase and leading to an increase in intracellular concentrations of the second messenger cAMP levels when stimulated with dopamine (Gingrich and Caron, 1993, Mustard et al., 2003, Sanyal et al., 2004). In mammals, $D_{1/}$ D_{1A} and D_{1B}/D_5 receptors constitute the D1-Like class that has been distend to include further subtypes in other vertebrates such as D_{1C} and D_{1D} (Sugamori et al., 1994, Demchyshyn et al., 1995, Cardinaud et al., 1997, Le Crom et al., 2004) . On the other hand, D2-Like receptors inhibit adenylyl cyclase or couple to second messenger signaling via inhibitory G protein (G_i) and modulate ion channels (calcium and potassium) (Gingrich and Caron, 1993, Jackson and Westlind-Danielsson, 1994). Members of D2-Like class are: the D2-, D3-, and D4-receptors (Missale, 1998, Andersen et al., 1990, Niznik and Van Tol, 1992). D2-like dopamine receptor subtype (D_2 receptor) have been described as $D_{2Short(S)}$ and $D_{2Long(L)}$ isoforms. Detailed molecular characteristics of mammalian dopamine receptors are presented in table 1.1.

Table 1.1 Molecular characteristics of mammalian dopamine receptors

	D_1-like		D_2-like		
	D_1	D_5	D_2	D_3	D_4
Chromosomal location on human genome	5q35.1	4p16.1	11q23.1	3q13.3	11p15.5
Gene Symbol	DRD1	DRD5	DRD2	DRD3	DRD4
Molecular weight	49.300	52.951	D_{2S}, 47.347; D_{2L}, 50.619	44.225	41.487
Amino acids	446 (h) 446 (r)	477 (h) 475 (r)	D_{2S}, 414(h); 415(r) D_{2L}, 443 (h);	400 (h) 446 (r)	387-515 (h) 385 (r)
Amino acids in 3rd cytoplasmic loop	57 (h) 57 (r)	50 (h) 50 (r)	D_{2S} ,134 (h); 135 (r) D_{2L}, 443 (h); 444 (r)	166 (h) 120 (r)	101-261 (h) 106 (r)
Amino acids in COOH terminal	113 (h) 113 (r)	116 (h) 117 (r)	16 (h) 16 (r)	16 (h) 16 (r)	18 (h) 18 (r)
Introns	0	0	D_{2S}, 5; D_{2L}, 6	5	3
G protein coupling	$G\alpha_s$, $G\alpha_{olf}$	$G\alpha_s$, $G\alpha_q$	$G\alpha_i$, $G\alpha_O$	$G\alpha_i$, $G\alpha_O$	$G\alpha_i$, $G\alpha_O$

r, Rat; h, Human

The table is compiled from information presented in review articles (Niznik and Van Tol, 1992, Sibley and Monsma, 1992, Sokoloff *et al*., 1992, Civelli *et al.*, 1993, Missale, 1998, Vallone *et al*., 2000, Seeman, 2006).

1.5.2 Structure of Dopamine receptors in vertebrates

Detailed analysis of crystal structure of dopamine receptors (Figure 1.2) reveals similarities and dissimilarities between D1-Like and D-Like dopamine receptor classes (O'Dowd, 1993, Jackson and Westlind-Danielsson, 1994). For D1-Like receptors the COOH-terminus length is about seven times longer than D2-Like receptors. Major difference between the two dopamine receptor classes is that the third intracellular loop is shorter in D1-Like receptors than in D2-Like (Probst et al., 1992, Civelli et al., 1993). Third intracellular loop is mainly responsible for G-protein coupling and signaling and specific regions of interactions lie near the N- and C- terminal regions of the loop (Macey et al., 2004, Johnston and Siderovski, 2007). The 3rd shorter intracellular loop in D1-Like receptor interacts with stimulatory G-proteins and the larger loop in D2-Like receptors coupled with inhibitory G-proteins (Missale, 1998).

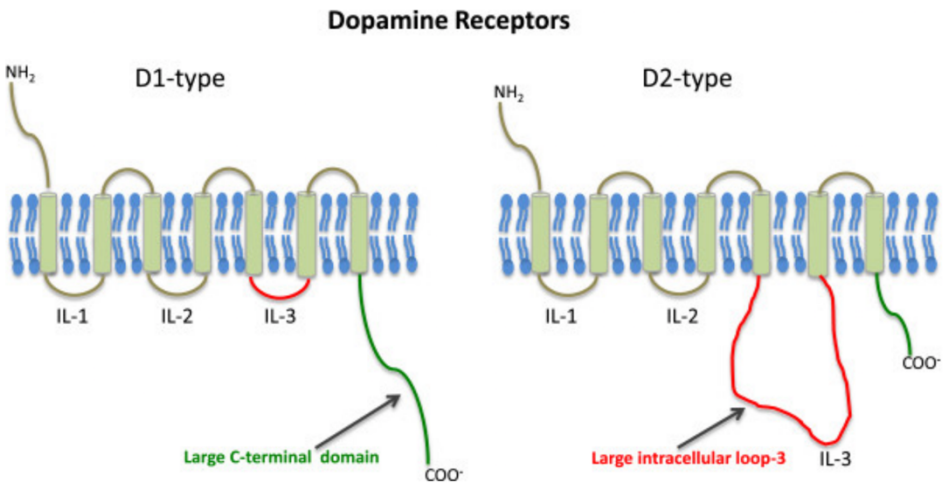


Figure 1.2: General difference between D1 and D2-like dopamine receptors structure. General structural differences between D1 and D2-like dopamine receptors are highlighted. Commonly observed seven transmembrane domains are represented with cylinders. D1-like dopamine receptor is shown with longer C-terminal (highlighted in green with arrow) and short 3rd intracellular loop while, D2-like dopamine receptor is represented with short COOH-terminus and longer 3rd intracellular loop (IL-3) (Pandey et al., 2013).

Individual members of the same class share wide range of DNA and amino acid sequence identity of their transmembrane domains and have different pharmacological properties. Genomic organization of mammalian D1 and D2-Like dopamine receptors can be differentiated by the presence and absence of introns in their coding sequences. D1-Like dopamine receptors (D1 and D5) do not contain introns in their coding regions, while introns are present in abundance in the genes that encode D2-Like receptors (Beaulieu and Gainetdinov, 2011).

In terms of genetic and structural characteristics, D1-Like and D2-Like dopamine receptors are distinct with respect to sequence homology. Mammalian D1 and D5 dopamine receptors share 80% identity in their transmembrane domains, whereas D2 and D3 receptors share 75% identity in their TM domains and D2 and D4 receptors are 53% identical. The NH_2-terminal region has similar number of amino acids in all of the dopamine receptor subtypes but differs in the number of consensus N-glycosylation sites, D1 and D5 possess two sites and D2 with four, D3 with three and D4 has only one site (Civelli et al., 1993, Gingrich and Caron, 1993).

In particular, highly conserved aspartate (Asp) residue in TM 2 and TM3 has been highlighted to play a vital role in D1 and D2 dopaminergic receptor activation and in binding the amine group of catecholamine side chain respectively. Two serine residues in TM 5 have been shown to be hydrogen bond donors that bind to the hydroxyl groups of the catechol moiety of D1 receptors. A phenylalanine residue in TM 6 is conserved in all receptors, interacts with catecholamine neurotransmitters and can make stabilized orthogonal interaction with the aromatic moiety of the ligand (Hibert et al., 1993, Missale, 1998) (Figure 1.3).

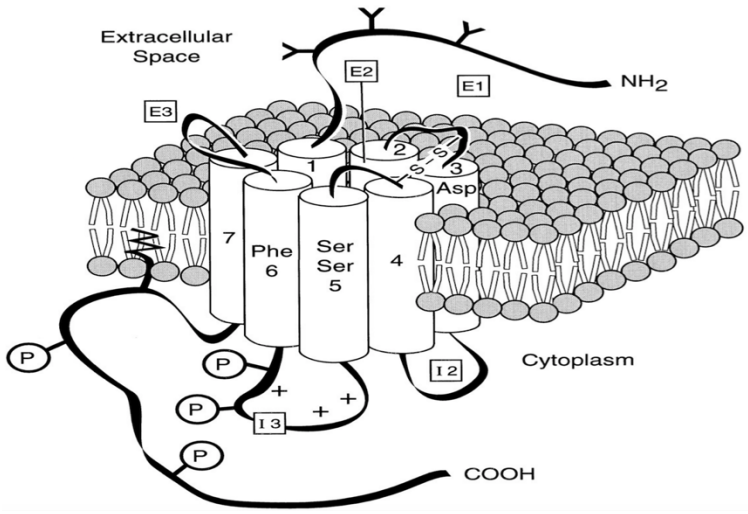


Figure 1.3: Conserved residues in dopamine receptor structure. Structural characteristics of D1-Like receptors are represented. Seven transmembrane domains are shown as alpha helical structures and the residues are highlighted in transmembrane domains that involved in dopamine binding. Extracellular (E1-E3) and intracellular loops (I1-I3) are shown with squares. Potential glycosylation sites are indicated on NH2-terminal. While, potential phosphorylation sites are represented on COOH terminus and 3rd intracellular loop (I3) (Missale, 1998).

In humans, two types of D_1-like receptors have been described such as D_{1A} (D_1), D_{1B} (D_5), while D_2-like receptors with $D_{2Short(S)}$ and $D_{2Long(L)}$ isoforms, D_3, and D_4 receptors. Central nervous system and peripheral blood lymphocytes express dopamine receptors in human. The predicted 3-dimensional structure of long isoform of human D2 dopamine receptor is shown in Figure 1.4.

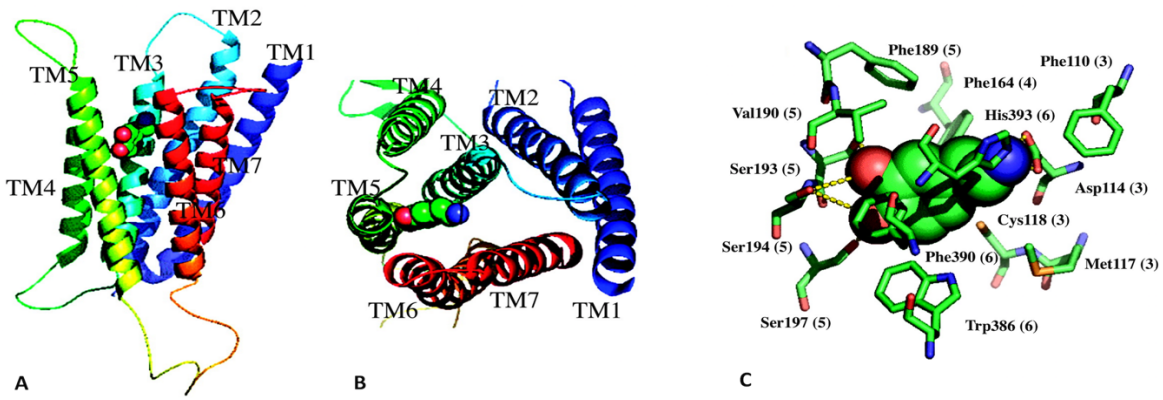


Figure 1.4: Three dimensional structure of dopamine from human D2-like receptor. Figure (A) shows a side view and Figure (B) shows the top view of the long isoform of the D2 human dopamine receptor. The ribbons show the alpha-helical transmembrane segments, while binding site of dopamine is shown in spheres. Favorable predicted binding site of dopamine is indicated in the top third of 7-TM barrel including TM domains 3-6. Figure (C) shows the residues with 5.5 Å of dopamine bound to human D2 dopamine receptor. The numbers shown in parentheses are the transmembrane helix to which the residues belong. Ser-197 on TM 5 makes a 2.7 hydrogen bond with parahydroxyl group, and Ser-193 makes a 2.7 hydrogen bond to the metahydroxyl group of dopamine (Kalani et al., 2004).

1.5.3 Distribution of dopamine receptors in vertebrates

Dopaminergic neurons are found mainly in the substantia nigra parscompacta, hypothalamus and in the ventral tegmental areas which give rise to three main pathways, the mesolimbic, the nigrostriatal, and the tuberoinfundibular. D1 receptors are widely distributed and highly expressed than any other dopamine receptors. It is found in the olfactory tubercle, striatum and nucleus accumbens, also in the thalamus, hypothalamus and limbic system. On the other hand, D5 receptor is expressed at lower level than D1 and is distributed to the hippocampus, the parafascicular nucleus of the thalamus and the lateral mammillary nucleus (Tiberi et al., 1991; Meador-Woodruff et al., 1992). D2 receptors are similarly expressed in the brain as D1 receptors. D2-like dopamine receptor subtypes including D3 and D4 have been found in the limbic areas and basal ganglia respectively. Besides that, D1 and D2 dopamine receptors have been localized in the renal and cardio-pulmonary system.

1.6 Signal Transduction Pathway

Dopamine receptor transduce signals by coupling to G-proteins and second messenger pathways (including cAMP, calcium, potassium, and AA) has been a subject of great interest (Figure 1.5). G-proteins composed of α, β and γ subunits which participate in several cellular activities such as development and signaling (Missale, 1998, Girault and Greengard, 2004). Activity of adenylyl cyclase is influenced by D1-Like and D2-Like dopamine receptors. D1-Like family receptors transduce signals by coupling to stimulatory G proteins (Gαs) and a closely related Gαolf (G-protein involved in olfaction), which thereby activate adenylyl cyclase (AC), leading to an increase in intracellular concentrations of the second messenger cAMP levels when stimulated with dopamine. D5 dopamine receptor and cloning of two non-mammalian D1-like receptor subtypes have been reported to be coupled to stimulation of AC, suggesting that the activation of AC seems to be general property of all D1-like receptors (Sunahara et al., 1991, Missale, 1998, Neve et al., 2004). On the other hand, D2-Like receptors inhibit the adenylyl cyclase activity or couple to second messenger signaling via inhibitory Gαi/o class of G-proteins and modulate ion channels (calcium and potassium). Adenylate cyclase further activates protein kinase A (PKA) which results in phosphorylation of downstream effector molecules (Na+/H+ exchangers, Na+-K+-ATPase), some of which influence gene expression. D1-like receptors modulate intracellular calcium levels via the stimulation of phosphatidylinositol hydrolysis by phospholipase (PLC). D2-like receptor cause the potential release of AA (Arachidonic Acid) evoked by calcium ions (Huff, 1996, Sidhu et al., 1998, Missale, 1998, Pandey et al., 2013).

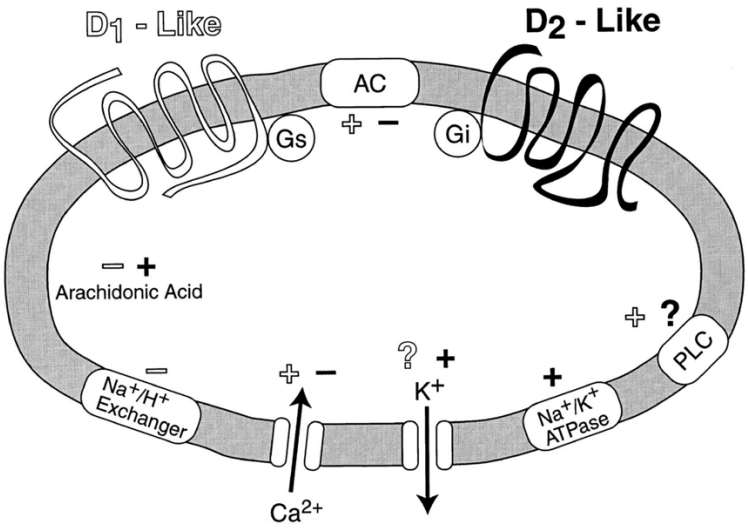


Figure 1.5: Signaling mechanism of dopamine receptor. Signal transduction pathway by D1-like and D2-like dopamine receptor is represented, involving second messengers such as adenylate cyclase (AC), phospholipase C (PLC), arachidonic acid and effector molecules (Missale, 1998).

1.7 Dopamine receptors in Arthropods

Dopamine receptors have been studied extensively in mammals, some insects and crustaceans. In arthropods, dopamine and its receptors plays an essential role for complex behavioral mechanisms such as arousal, locomotion and olfactory learning and control many vital biological processes such as development, terminal differentiation of nervous system, metabolism and signaling process (Nassel and Elekes, 1992, Yellman et al., 1997, Kume et al., 2005, Draper et al., 2007, Kim et al., 2007, Riemensperger et al., 2011, Mustard et al., 2010). The biological mechanism of dopamine receptors in *L. salmonis* is not known at the molecular level. For this reason, insects such as Drosophila and ticks functions as a model organism to gain insights into the function, mechanism and regulation of dopamine receptors that can further be utilized to understand the molecular biology of the salmon louse.

Ticks are obligate blood-feeding ectoparasites comprised of Argasidae and Ixodidae families. Salivary glands are important and major route of pathogen transmission in ticks, and dopamine receptor act as an autocrine/paracrine activator of salivary secretions (Sauer *et al*., 2000, Simo *et al*., 2014). In ticks, dopamine receptor of type D1 is found which is highly expressed in salivary glands. The salivary glands of ticks have different functions as excess fluid excretion for blood

meal concentration, removal of excess water and ions by free-living ticks and secretion of bioactive proteins and lipids during feeding. These proteins and lipids are necessary in order to fix the mouth parts to the host skin, for osmoregulation during feeding, and contain anti-inflammatory, antithemostatic, and immunomodulatory substances. It has been proposed that dopamine receptor acts over two independent signaling pathways in ticks, calcium–dependent signaling pathway that activates prostaglandin E2 production which leads to the secretion of other components in the saliva and cAMP-dependent signal transduction leads to the fluid secretions. Prostaglandin E2 is highly secreted into tick saliva for export to the host where it effects the host physiology (Sauer et al., 2000, Šimo et al., 2011, Šimo et al., 2012).

Dopamine is a biogenic amine with relatively high concentrations in the insect nervous system, where the dopaminergic neurotransmissions modulates learning, memory and neuronal development (Tempel *et al*., 1984, Budnik *et al.*, 1989, Buchner, 1991, Reale *et al*., 1997). In flies dopamine regulates sexual function and response to drugs (Yellman *et al*., 1997, Li *et al*., 2000). In Drosophila, D1-like and D2-like dopamine receptors with multiple isoforms due to alternative splicing have been characterized. D2-like receptor is shown to be expressed during larva and pupa development as well as in the adult fly and DD2R transcript is most abundant in *Drosophila* adult head (Gotzes *et al*., 1994, Hearn *et al*., 2002). In addition, several studies confirmed the presence of D1-and D2-like dopamine receptors in *Caenorhabditis elegans* with D2 receptor splice variants (Suo *et al*., 2002, Suo *et al*., 2003). Many sites are found in the brains of honey bee and cockroach for D1-like dopamine receptor (Macrae and Brenner, 1995, Kokay and Mercer, 1996, Hirano *et al*., 1998).

In previous studies, presence of prostaglandin E synthase of *L. salmonis* has been reported in adult *L. salmonis* secretions, when stimulated by neurotransmitter dopamine (Fast et al., 2004). These secretions are believed to have an anti-inflammatory and immuno-suppressive role in *L. salmonis* that feeds on a host (Fast et al., 2007, Wagner et al., 2008). It will be an interesting endeavor to identify and gain an in-depth knowledge about dopaminergic pathways in *L. salmonis* which can serve as targets to control the parasite infections.

1.8 Aims of the study

The aim of the present study is to investigate the function of D1-like and D2-like dopamine receptors in *L. salmonis* by the use of different molecular techniques. In ticks, dopamine receptor of type D1 has been shown to be involved in salivary secretions and for *L. salmonis* it has been proposed that dopamine acts in a similar way as in ticks. The main objectives are to:

- Characterize the gene structure, domain organization and phylogeny using bioinformatics tools
- Clone and sequence the *L. salmonis* dopamine receptor gene, LsDopamine1
- Identify the expression and localization by *in situ* hybridization
- Gene knock down of LsDopamine1-2 by RNAi
- Confirm knock down of *L. salmonis* genes by qRT-PCR

2 Materials

2.1 Chemicals

Table 2.1 Chemicals used

Chemical	Formula	Supplier
100 % Ethanol	C_2H_6O	Sigma-Aldrich
96 % Ethanol	C_2H_6O	Sigma-Aldrich
Acetic anhydride	$C_4H_6O_3$	Sigma-Aldrich, USA
BCIP 4-toluidine salt	$C_8H_6BrClNO_4P$ x C_7H_9N	Roche Diagnostics, Germany
Chloroform	H_3BO_3	Sigma-Aldrich, USA
Deionized formamide	CH_3NO	Sigma
Diethyl pyrocarbonate (DEPC)	$C_6H_{10}O5$	Sigma
Ethylene-diamine-tetra-acetic acid (EDTA)	$C_{10}H_{16}N_2O_8$	Sigma-Aldrich, USA
Ethidium Bromide	EtBr	Sigma-Aldrich
GelRed 10000X		Biotium, Inc., USA
Histoclear	C_1H_{16}	Chemie Teknik
Isopropanol	C_3H_8O	Kemetyl Norge AS
Lithium Chloride	LiCl	Merck, Germany
Magnesium chloride x 6H2O	MgCl2.6H2O	Merck
Maleic aciid	$C_4H_4O_4$	Sigma-aldrich
Metamidate	$C_{13}H_{14}N_2O_2$	Aqua Qualm
Sodium chloride	NaCl	Merck, Germany
Sodium hydroxide	NaOH	Merck, Germany
NBT (4-Nitro blue tetrazolium)	$C_{40}H_{30}C_{12}N_{10}O_6$	Roche Diagnostics, Germany
Triethanolamine (TEA)	$C_6H_{15}NO_3$	Sigma-aldrich, USA
Tris base (Tris-(hydroxymethy)-aminomethan	$C_4H_{11}NO_3$	Merck, Germany
Triton X-100	$C_{16}H_{26}O_2$	Sigma-Aldrich
Tween 20 (Polyxyethylenesorbitan)	$C_{58}H_{114}O_{26}$	Sigma

All chemicals used were chemically pure of P.A. grade.

2.2 Solutions and Compounds

Table 2.2: Solutions used

Name	Supplier
Agar-Agar	Merck
Agarose	Lonza, USA
Bacto Trypton	Bacto, Dickinson and company (BD)
Bacto Yeast Extract	Bacto, Dickinson and company (BD)
Blocking reagent	Roche
Dextran sulphate	Sigma-Aldrich
Gel loading Dye Blue 6x	New England Biolabs
GenElute™ LPA	Sigma-Aldrich
Deoxyribonucleotide phosphate (dNTP)	Promega, USA
Paraformaldehyde	Sigma
RNA later	Qiagen, USA
Trizol reagent	Sigma-Aldrich

2.3 Antibiotics

Table 2.3: Antibiotic used

Name	Supplier
Ampicillin	Bristol-Meyers- Squibb

2.4 Enzymes

Table 2.4: Enzymes used

Enzymes	Supplier
DNA polymerase I	Promega
DNAse I	Invitrogen, USA
Proteinase K	Sigma-Aldrich, USA

2.5 Antibody

Table 2.5: Antibody used

Name	Supplier	Catalogue Number
Anti-Digoxigenin-AP Fab fragment n (Fab fragments from sheep)	Roche	11207741910

2.6 Consumables

Table 2.6: Consumables

Name	Supplier
1.4 mm Zirconium oxide beads	Precellys, Bertin Technologies
5 mm stainless steel beads	Qiagen
1.5 ml Eppendorf tube	Eppendorf
15 ml reaction tube	Cellstar® greiner bio-one
50 ml reaction tube	Sarstedt
3 MM Whatman 20x20 cm	VWR
Microamp® FAST Optical 96-well reaction plate	Applied Biosystems, USA
Plastic seals for qPCR	Abgene™
Petri-dish (100 ml)	Sarstedt
Pipette tips	Axygen Scientific
Cover glass (24 x 60 mm)	Menzel-Glaser
Hybond N+	VWR
PAP penn	DAKO

2.7 Molecular Biology Kits

Table 2.7: Molecular Biology kits used

Kit	Supplier
AffinityScript QPCR cDNA Synthesis Kit	Agilent Technologies
BigDye® Terminator v3.1 Cycle Sequencing kit	Applied Biosystems
DIG RNA labelling Kit	Roche
GoTaq® Flexi DNA Polymerase	Promega, USA
MEGAscript® RNAi Kit	Applied Biosystems/ Ambion, USA
NucleoSpin® Gel and PCR Clean-up kit	Macherey-Nagel
NucleoSpin® Plasmid, Nucleic Acid and Protein Purification Kit	Macherey-Nagel
qScript cDNA SuperMix	Quanta Bioscience, USA
SMARTer™ RACE cDNA Amplification Kit	Clontech
TOPO TA Cloning® Kit for Sequencing	Invitrogen, USA
UltraClean® 15 DNA Purification Kit	MO BIO

2.8 Equipments

Table 2.8: Equipements used

Equipment	Purpose	Supplier
7900 Fast Real-Time PCR system	RT-qPCR	Applied Biosystems, USA
Camera: Lieca DFC420	Obtaining images from in situ slides	Leica Microsystems
GelLogic 212 PRO	Visualize GelRed stained DNA bands	Fisher Scientific
GelDoc™ EZ Imager	Visualize EtBr stained DNA bands	BIO-RAD
Gel Image Printer	Print agarose gel image	Mitsubishi P93D
GenAmp PCR system, 9700	Polymerase Chain Reaction	Applied Biosystems, UK
GenAmp PCR system, 2700	Polynerase Chain Reaction	Applied Biosystems, UK
HII 210 (bath container)		Leica, Germany
HERAEUS FRESCO 21 Centrifuge	Centrifugation	Thermo Scientific
Incubator 37 °C	Incubation and growth of transformed bacterial cells in petri-plates	Termaks
Incubator 37 °C , 250 rpm	Bacterial cell growth	Tamro MED-LAB
Incubator 60 °C	Incubation of *in situ* slides	Thermo Hybaid Shake 'n' Stack
Light cycler® 480 QPCR machine	qRT-PCR	Roche
Milli-Q Advantage A10, Milli-Q Q-POD®, 0.22 m Milli PAK® 40 sterile	Mili-Q water	MILLIPORE Lab-tec
Microscope: Olympus SZX9	Visualizing sea lice	Olympus
Nanodrop ND-1000	Measuring DNA, RNA concentration	Thermo Scientific, USA
Thermal Cycler, Verti 96 Well	Mixing and spin down	Applied ssytems
Tissue Lyser LT	Homogenizing sea lice	Qiagen

2.9 Molecular Size Marker and Bacterial strain

Table 2.9: Molecular size marker used

Name	Range	Supplier
2-Log DNA Ladder	0.1-10.0 Kb	TaKaRa
MassRuler DNA ladder mix	0.08-10.0 Kb	Fermentas, Canada

Table 2.9.1: Bacterial strain used

Name	Purpose	Supplier
One Shot® TOP10 *E. coli*	Transformation	Invitrogen

2.10 Primers

Table 2.10 Primers used for PCR

Primer	Sequences (5' - 3')	Supplier
b1751 Forward	GGGGCCTACCAAAGATTGCA	Sigma-aldrich, USA
b1758 Reverse	GCCCCAATAATTTCTTGAATGCC	Sigma-aldrich, USA
b3464 Forward	TCCCACATTTAAACGGGGCTATT	Sigma-aldrich, USA
b3465 Reverse	AAATTGCCGGATTCATTCCCGA	Sigma-aldrich, USA
b3479 Forward	GGGCCCTGCTTCTATTATTGC	Sigma-aldrich, USA
b3480 Reverse	GCTAGATGTAGGGGAAGATGGTG	Sigma-aldrich, USA
b3481 Forward	GTTGCTCGAGAAAAGGTTCTGC	Sigma-aldrich, USA
b3482 Reverse	GGAGTTGATATATCCAAGCCAGGT	Sigma-aldrich, USA
b3771Reverse	GTCCAAGCCCCAATAATTTCTTGAATGCC	Sigma-aldrich, USA

Table 2.10.1: Primers used for RACE and Sequencing

Primer	Sequences (5' - 3')	Supplier
M13-Forward	AACGACGGCCAGTGAATTGT	Invitrogen, USA
M13-Reverse	ATGACCATGATTACGCCAAG	Invitrogen, USA
b3547 Forward	TGGGGAATGTGGGGCCTACCAAAGATTGC	Sigma-aldrich, USA
b3629 Forward	TGGCTCACCTGTCATGCTAGG	Sigma-aldrich, USA
b3770Forward	ACCATCTTCCCCTGTACCAACAAAGCCA	Sigma-aldrich, USA
b3783Reverse	CCCACATTCCCCAATTAACCAGCAC	Sigma-aldrich, USA
b3784Reverse	CAGAAAAGGGCATTACAAATCCGGCTAC	Sigma-aldrich, USA

Table 2.10.2: Primers used for SYBR Green assay

Primer	Sequences (5' - 3')	Supplier
b3483 Forward	GGCGGGATAACTCCCAAAAG	Sigma-aldrich, USA
b3484 Reverse	CCCAGCCACGTTACAACT	Sigma-aldrich, USA
b3731 Forward	CCTGTCATGCTAGGCGCTAA	Sigma-aldrich, USA
b3732 Reverse	TGATCCAAGCGACGAATAAATG	Sigma-aldrich, USA

2.11 Databases and Software

Table 2.11: Databases used

Name	Web Address
GenBank	http://www.ncbi.nlm.nih.gov/genbank/
LiceBAse	https://licebase.org/
NCBI Primer BLAST	http://www.ncbi.nlm.nih.gov/tools/primer-blast/
NCBI BLAST blastn	http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE =BlastSearch&LINK_LOC=blasthome
NCBI BLAST blastp	http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch& BLAST_SPEC=&LINK_LOC=blasttab&LAST_PAGE=blastn
SMART	http://smart.embl-heidelberg.de
Uniprot	http://www.uniprot.org

Table 2.11.1: Software used

Name	Web Address
Clustal Omega	http://www.ebi.ac.uk/Tools/msa/clustalo/
Clustal X	http://www.clustal.org/clustal2/
ExPASy	http://web.expasy.org/translate/
GIMP	https://www.gimp.org/downloads/
ImageJ 1.49 v	https://imagej.nih.gov/ij/download.html
I-TASSER	http://zhanglab.ccmb.med.umich.edu/I-TASSER/
MEGA7 version 7.0.1	http://www.megasoftware.net
N-J Plot	http://pbil.univ-lyon1.fr/software/njplot.html
Staden package	http://staden.sourceforge.net/

3. Buffers, Media and Solutions

3.1 General Solutions and Media

Agarose gel 1%
1 % Agarose in 1 X TAE

50 X TAE (Tris-Acetate-EDTA) buffer
57 ml Glacial acetic acid
100 ml 0.5 M EDTA pH 8.0
ddH2O upto 1000 ml

1 X TAE (Tris-Acetate-EDTA)
20 ml 50 X TAE buffer
ddH2O upto 1000 ml

LB (Luria-Bertani) Medium
1 % Bacto trypron
0.5 % Bacto Yeast Extract
0.5 % Sodium chloride
Autoclaved before use

Agarose-TBE gel (1%)
1 % Agarose in 0.5 x TBE
0.5 µg/ µl EtBr

5 X TBE
0.45 M Trisma base
0.45 M Boric acid
0.01 M EDTA

Loading buffer (6x)
0.25 % Bromophenol blue
40 % sucrose
Milli-Q water

LB-Agar plate
1 % Bacto trypron
0.5 % Bacto Yeast Extract
0.5 % Sodium chloride
1.5 % Agar-Agar
Autoclaved before adding ampicillin
100 µg/ µl Ampicillin

3.1.1 Solutions and buffers for *In situ* hybridisation

Washing Bufer A
10 ml 5x Maleate buffer
40 ml DEPC
150 µl Tween 20

Detection buffer A
5 ml 1 M Tris HCl
5 ml 5 M NaCl
40 ml DEPC
Adjust pH to 9.5

1 % blocking solution A
10 ml 5x Maleate buffer
40 ml DEPC
5 ml 1 % Blocking solution

Blocking solution 10 %
10 g blocking reagent
100 ml maleic buffer
slowly dissolved by heating
store at -20 °C

DEPC water
1 ml Diethylpyrocarbonate
1000 ml MilliQ water
Incubate at 37 °C overnight
Autoclave

Deionized formamide
store at -20 °C

Hybridization solution
2.5 g dextran sulphate
DEPC H2O upto 5 ml
250 µl 1 M Tris HCl, PH 7.5
50 µl 0.5 M EDTA
1.5 ml 5 M NaCl
0.7 ml DEPC H2O
12.5 ml deionized formamide
store at -20 °C

Maleate buffer 5x
58 g Maleic acid
850 ml MilliQ water
Adjust pH to 7.5 using NaOH pellets (app. 35 g to 11.)
43.8 g NaCl
Milliq water upto 1L
Store at RT

MgCl2 stock
4.65 g 0.5M MgCl2
100 ml DEPC H2O

PBS (1x) Phosphate Buffered Saline
Tablets
4X PBS

Processing buffer
100 mM Tris-NaCl pH 9.5
50 mM MgCl2
DEPC H2O upto 1000 ml

4% Paraformaldehyde in PBS
40 g Paraformaldehyde
500 ml DEPC treated water
150 µl 1 M NaOH (heat upto 65 °C untill PF is dissolved)
Cool to room temperature
100 ml 10X PBS
Adjust pH to 7.4, volume 1000ml
store at -20 °C

Rnase buffer
29.23 g 0.5M NaCl
10 ml 1M Tris HCl pH 7.5
2 ml 0.5 M EDTA
DEPC H_2O upto 1000 ml

SSC buffer (20X)
175.3 g NaCl
88.2 g sodiumcitrate
800 ml DEPC dH2O
adjust pH 7.0 with NaOH
DEPC H2O upto 1000 ml

Tris HCl pH 7.5
121.1 g Tris base
800 ml DEPC dH2O
Adjust pH to 7.5 with HCl
DEPC H2O upto 1000 ml

Stop buffer
10 mM Tris-HCl pH 7.5
1 mM EDTA
150 mM NaCl

Tris NaCl stock pH 9.5
60.55 g Tris base
350 ml MilliQ water
29 g NaCl
Adjust pH to 9.5 using HCl
MilliQ H2O upto 500 ml

4. Methods

4.1 Bioinformatics Analysis

4.1.1Database search and verification

LiceBase is a database comprising the genome sequence of *Lepeophtheirus salmonis* as well as predicted genes and protein sequences. FASTA sequences of dopamine receptors from other species were selected using GenBank database. BLAST search with these selected sequences were then carried out against the salmon louse genome to obtain the homologous sequences in *L. salmonis*. Eight genes were selected from LiceBase and NCBI BLAST search against these *L. salmonis* sequences gave hits with dopamine receptor for three genes i.e. EMLSAG00000003021, EMLSAG00000003269 and EMLSAT00000003268 which were named as LsDopmaine1 (EMLSAG3021) and LsDopamine2 (EMLSAT00000003268-EMLSAG00000003269). In LiceBase, the predicted dopamine receptor cDNAs of *L. Salmonis* EMLSAT00000003268 and EMLSAG00000003269 were found on the same contig. There was found a stop codon in the EMLSAG00000003269 sequence and BLAST search of EMLSAT00000003268 and EMLSAG00000003269 also displayed partial receptor sequences. To verify if EMLSAT00000003268 and EMLSAG00000003269 are parts of the same gene, sequence analysis was performed.

4.1.2 Domains and structure prediction

LsDopamine1 and LsDopamine2 nucleotide sequences were translated to amino acid sequences with ExPASy tool. The domain prediction of LsDopamine1 and 2 protein sequence was made in SMART tool and structure prediction was performed using I-TASSER protein structure and function predictions approach. Sequence of the D1-like dopamine receptor in *Aedes aegypti* and D2-like dopamine receptor in *Drosophila melanogaster* was used to localize domains and to identify and compare conserved structural features.

4.1.3 Phylogenetic tree construction

The evolutionary study of relationships among different species that have descended from a common ancestor is called phylogeny. The inferred relationship is represented by phylogenetic tree based on similarities and differences in their genetic and physical characteristics. In phylogenetic tree, each species is represented as a node and the relationship between species is represented as a branch and the branch lengths indicate changes over time (Yang and Rannala, 2012). Two common approaches Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and N-J (Neighbor Joining) plot based on clustering method can be used to build an evolutionary tree. Bootstrapping is a re-sampling analysis from the existing samples with replacement and a common method to assess the reliability of the reconstructed phylogenies. This method tests to recover the same nodes by taking 100 or 1000 iterations and a bootstrap value of ≥ 70% is considered a good support for each clade of the reconstructed tree (Hillis and Bull, 1993, Efron et al., 1996).

To investigate the evolutionary relationship between LsDopamine1-2 and other homologous sequences from different species, a phylogenetic analysis was performed. To construct a phylogenetic tree, the amino acid sequences of 29 D1 and D2-like dopamine receptors from different species of vertebrates and invertebrates and of octoapamine and serotonin receptors were taken by performing searches in GenBank (NCBI) and Uniprot databases. Multiple sequence alignment was performed in Clustal X v 2.1 with Gap Opening 10, Gap Extend, 0.1, Protein Weight Matrix (BLOSUM 30) and DNA Weight Matrix (IUB) and looked for similarities and the conserved regions. The phylogenetic tree was constructed using neighbor joining (N-J) method with bootstrap values and the basic parameters were random number generator seed 111 and 1000 number of bootstrap trials.

4.2 Sea lice sampling and photographic documentation

All the sampling of sea lice was performed in Fish laboratory of sea lice research center (SLRC). For this egg strings from female salmon lice were hatched and kept in hatching wells until copepodid stage. The sea lice strains in laboratory were raised in sea water with salinity of 34% and 10 °C temperature with farmed Atlantic salmon (*Salmo salar)* which were fed on commercial diet (Hamre et al., 2009). All procedures were performed according to the Norwegian animal welfare regulations.

4.3 Analysis of DNA sequences

4.3.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (Mullis, 1990) is a simple method, which allows for the amplification of a specific gene sequence of interest from a minute amount of starting material in a cycling process. PCR is divided into three steps of repetitive cycling includes i) denaturation, where temperature is raised to open up the double stranded DNA ii) annealing, the temperature is lowered to allow the annealing of primers iii) elongation, the increase in temperature let the polymerase extend the primers by incorporating dNTPs. Primers are the defined sequence complementary to the target DNA and specify the amplification of exact DNA product (Kubista et al., 2006, Garibyan and Avashia, 2013). Melting temperature of the primers was determined in primer-BLAST at NCBI. The annealing temperature was estimated by subtracting 5 °C from the melting temperature (Tm°) of the given primers. The elongation time was set to 1 min/1000 bp of PCR product.

PCR was performed using Go Taq flexi DNA polymerase kit in order to detect the size of DNA fragments from agarose gels. 1 µl of the synthesized cDNA was used as a template for a standard PCR reaction (Section 4.6.1). Master mix was prepared with 5X Green Go Taq Flexi Buffer (5x), $MgCl_2$ solution 2.5 mM, dNTPs 1.25 mM, Go Taq Flexi DNA polymerase 1U and nuclease-free water up to a desired volume. PCR reactions were carried out in a final volume of 25 µl, with 10 µM primer (forward and reverse), and 1µl template (synthesized cDNA). The PCR was carried out in a 2700 PCR system (Applied Biosystems) (Table 4.1)

Table 4.1: Amplification conditions of PCR

Temperature	Time	
94 °C	2 min	
94 °C	30 sec	35 cycles
55-62 °C	15 sec	
72 °C	1min/1000 bp	
72 °C	5 min	
4 °C	Hold	

4.3.2 Rapid Amplification of cDNA ends (RACE)

SMARTer™ RACE cDNA Amplification kit was used for 5' - and 3' rapid amplification of cDNA ends (RACE) to obtain full length cDNA sequence produced through reverse transcription (Zhu et al., 2001). Amplification of cDNA ends was carried out with kit primers and LsDopmaine2-specific primers. The PCR reaction was carried out in a final volume of 25 µl, with 1.25 µl template, 0.5 µl 10 µM Gene specific primer (forward or reverse), 2.5 µl 10 x Advantage 2 PCR buffer, 0.5 µl 10 mM dNTPs, 2.5 µl 10 x UPM, NUP A (1 µl for final volume of 50 µl reaction) and 0.5 µl 50 x Advantage 2 Polymerase mix and water up to a desired volume. PCR reaction was run with 35 cycles on 2700 PCR system (Applied Biosystems).

4.3.3 Agarose gel electrophoresis

Agarose gel electrophoresis (a method of separating biomolecules according to size) was used for purifying PCR products and analyzing genes of interest. 1 % agarose gel in 1 x Triethanolamine (TAE) buffer was prepared and run on 90 V current for 30 min. GelRed was added to the agarose to visualize the migration of DNA fragments in the gel. Gel Doc™ EZ imager (BioRad) was used for gel analysis.

As per requirements, the DNA was extracted and purified from agarose gel by following NucleoSpin Gel and PCR clean-up kit instructions. All the centrifugation steps were carried out at the speed of 14,000 x g in Heraesus Biofuge pico centrifuge. All impurities such as nucleotides, enzymes and salts from PCR reaction were washed by using the silica membrane. According to manufacturer's protocol, the DNA was eluted in 20 µl elution buffer NE provided in the kit.

4.4 Sanger sequencing of LsDopamine2 gene

For sequencing of LsDopamine2 gene, the PCR and RACE products were cloned into the PCR4-TOPO® vector for further analysis of size and variants of DNA fragments.

4.4.1 Cloning and cultivation of bacteria

TOPO® TA Cloning® Kit for Sequencing (Invitrogen) was used. Cloning reaction was carried out in a final volume of 6 µl with 4 µl purified PCR product, 1 µl salt solution and 1 µl TOPO® cloning vector. The reaction mixture was spun down and incubated for 15 min at room temperature. In order to transform the competent cells, 2 µl of TOPO cloning reaction was added to 40 µl competent *E. coli* cells, mixed gently by tapping and incubated on ice for 15 min. The cells were then heat shocked in a heating block at 42 °C for 30 seconds without shaking and immediately put the tubes on ice. 250 µl SOC-medium was added to the reaction mixture, followed by incubation at 37 °C by horizontally shaking for 1 hour, 250 rpm. Finally, 50 µl and 100 µl from the transformed bacteria were spread evenly on LB-agar plates containing ampicillin (100 µg/ml) to select for positive colonies. The agar plates were incubated at 37 °C overnight. Several colonies of bacteria were picked for further analysis using the colony PCR. Single colony culture containing LB-medium was used for small scale plasmid purification.

4.4.2 Plasmid DNA purification from *E. coli* (Mini-prep)

DNA mini prep was performed in order to isolate and purify plasmid DNA from bacteria. The NucleoSpin Plasmid Nucleic acid and protein purification kit and the respective protocol (Macherey-Nagel) were used for this purpose. By following the protocol, the plasmid DNA was eluted in 50 µl elution buffer AE. The concentration of purified plasmid was measured on Nanodrop and length was verified on 1 % agarose gel.

Prior to sequencing, the PCR products were purified by ExoSAP-it PCR product clean up reagent. PCR purification reaction was performed by taking 2.5 µl PCR product, 1 µl ExoSAP-it. The reaction mixture was mixed together and incubated in a PCR machine (Table 4.2).

Table 4.2 : Program for Exosap-it

Temperature	Time
37 °C	15 min
80 °C	15 min
4 °C	∞

After PCR clean-up, 5 µl of water was added in ExoSAP reaction and concentration was measured in Nanodrop (Nanodrop ND-1000).

4.4.3 Sanger sequencing using Big dye

Sequencing was performed on cDNA sequences of *L. salmonis* EMLSAG3269 and EMLSAG3268 in order to obtain full length sequences and to characterize exon-intron pattern. In Sanger method, fluorescently labelled dideoxynucleotides (dNTPs) that lacks 5' hydroxyl group are used to synthesize PCR fragments which allows the formation of 5' to 3' phosphodiester linkage in the chain (Sanger et al., 1977). Linear amplification was achieved by carrying the sequencing reactions with forward and reverse primers separately. Sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) by preparing the master mix with 1 µl Big Dye, 1 µl sequencing buffer, 1 µM M13 primers (forward and reverse). X µl of plasmid (200-400 ng) and Milli-Q H_2O up to a final volume of 10 µl. The PCR was run in a 2700 PCR system (Applied Biosystems) (Table 4.3).

Table 4.3: Sequencing program

Temperature	Time	
94 °C	5 min	
94 °C	10 sec	27 cycles
50 °C	5 sec	
60 °C	4 min	
4 °C	Hold	

After the completion of cycling program, 10 µl Milli-Q H_2O was added to each of the reactions and sent to the sequencing facility of the University of Bergen. The sequencing data was further assembled and edited using Staden software. After comparing the sequence from cDNA and the genomic sequence from LiceBase, the exons and introns were assigned. Homology sequence analysis was performed using the Basic Local Alignment Search Tool (BLAST) at NCBI.

4.5 RNA extraction

Lice used for RNA extraction were stored in RNAlater. RNA was extracted using TRI reagent (Sigma-Aldrich) with some modifications adjusted by the Sea Lice Research Center (SLRC). The total RNA was purified from both the early developmental lice samples (nauplii and copepodids) and from individual lice samples (adult). For the early developmental stages (nauplii I, II and copepodids), 1 ml TRI reagent and salmon lice were added to an eppendorf tubes containing zirconium oxide beads and homogenized for 2 minutes at 50 Hz using Tissue Lyser LT (Qiagen). For later developmental stages (chalimus, preadult and adult), one adult male louse was added to an eppendorf tube containing one 5 mm stainless steel bead, and 1 ml TRI reagent by following homogenization for 5 minutes at 50 Hz. The homogenate was allowed to stand for 5 minutes at room temperature to ensure complete destruction of the tissue. After 5 minutes of incubation, 200 µl of chloroform was added to the homogenate followed by rigorous shaking for 15 seconds. The samples were then incubated for 15 minutes at RT. For upper colorless aqueous phase separation containing RNA from the rest two phases i.e. pinkish phase containing protein,

a white interphase containing DNA, the tubes were centrifuged at 12,000 x g for 15 minutes at 4 °C. Approximately 450 µl of the supernatant was transferred to a new eppendorf tube and 0.5 ml of isopropanol was added. Samples were mixed and allowed to stand for 10 minutes at room temperature followed by centrifugation at 12,000 x g for 10 minutes at 4 °C. The supernatant was discarded and RNA pellet was washed with 1 ml of 75 % ethanol. The samples were vortexed and centrifuged at 7,500 x g for 5 minutes at 4 °C. The supernatant was discarded and RNA pellet was washed with 500 µl of 75 % ethanol followed by centrifugation at 7,500 x g for 5 minutes at 4 °C. The elute was discharged and RNA pellet was dried for 5 minutes, before it became colorless, and 50 µl RNase free water was added. The purity of extracted RNA was checked by Nanodrop Spectrophotometer (Nanodrop ND-1000), by analyzing the absorbance ratio ($A_{260}\backslash A_{280}$ ratio) for which a value greater than 2.0 is considered as acceptable RNA quality and shape of curves (Wilfinger et al., 1997). RNA samples either were stored at – 80 °C until use or cDNA synthesis was performed directly.

4.6 cDNA synthesis

cDNA synthesis was carried out using different kits for standard polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR). Total RNA from each stage of the lice was used for cDNA synthesis.

4.6.1 cDNA synthesis for PCR

For standard polymerase chain reaction (PCR), cDNA synthesis was carried out using the qScript cDNA SuperMix Kit from Quanta Biosciences. A total of 20 µl reaction was prepared on ice by taking 1 µg/µl of total RNA, 4 µl qScript cDNA SuperMix (5X) and RNase/DNase-free water. The components were mixed and centrifuged briefly and run in a 2700 PCR system (Applied Biosystems) (Table 4.4).

Table 4.4: PCR cDNA synthesis program

Temperature	Time
25 °C	5 min
42 °C	30 min
85 °C	5 min
4 °C	∞

4.7 Two-step quantitative real-time polymerase chain reaction (qRT-PCR)

In order to eliminate DNA, total RNA was DNase treated prior to reverse transcription for the preparation of template cDNA for use in qRT-PCR. DNase treatment was carried out with AmpGrade, DNase I (Invitrogen). Negative purification control (-NC) was used to monitor the presence of contaminating RNA. For DNase treatment, a final volume of 10 µl reaction was made with 1 µg/µl of purified RNA, 1 µl 10X DNase I reaction buffer and 1 µl DNase I. DEPC-treated water was added up to a desired volume. The components were mixed and incubated for 15 min at room temperature. 1 µl of 25 mM EDTA was added and PCR tubes were incubated at 65 °C for 10 min. After DNase treatment, cDNA synthesis was performed by reverse transcriptase enzyme using AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies). A minus reverse transcription control (-RT) was also included to check the presence of contaminating DNA. Master mix was prepared with 5 µl of 2X First strand master mix, 10 µM oligodT primer, 10 µM random primer, 1 µl RNA, 1 µl H_2O. cDNA synthesis reaction was carried out in a final volume 10 µl with 0,5 µl affinity Script RT. The reaction mixture was spun down and run in a 2700 PCR system (Applied Biosystems) (Table). cDNA was diluted three to seven times before storage at – 20 °C.

Table 4.5: qRT-PCR cDNA synthesis program

Temperature	Time
5 °C	5 min
42 °C	15 min
95 °C	5 min
4 °C	∞

qRT-PCR is the most sensitive method which allows the detection and quantification of target cDNA molecule in real time after each cycle. As the qRT-PCR progresses, with the accumulation of product after each cycle the signal from a fluorescent reporter molecule increases and it is possible to determine the amount of fragment (Higuchi et al., 1992). Ideally, the signal is doubled after each cycle. qRT-PCR was performed to confirm the down regulation

of the target genes after RNAi. qRT-PCR was run on Light cycler® 480 QPCR (Rouche) using the Light cycler® 480 SYBR Green I Master mix. Master mix was prepared with 10 µl SYBR Green I Master mix, 3 µl H_2O and 1 µl 10 µM primer (forward and reverse). qPCR reaction was executed in a final volume of 20 µl with 5 µl template cDNA and run with 42 number of cycles.

In this study, a standard curve was established with eight dilutions of RNA to determine the primers efficiency for each assay. NTC (no template control) was used to monitor possible contamination. For obtaining robustness in data, qRT-PCR was carried out in triplicates and five biological parallels. A melting curve was run to monitor primer-dimer formation for SYBR green assays and relative quantification was performed using the ΔΔCt method (Livak and Schmittgen, 2001) to determine the changes in gene expression compared to EF1α as a reference gene. EF1α has been validated as a reference gene in different *L. salmonis* developmental stages (Frost and Nilsen, 2003). Ct values which represents the cycle number at which the fluorescent signal crosses the threshold line were calculated and normalized to EF1α. T-tests were conducted to determine the significant difference between expression levels in RNAi lice and control samples. A p-value of 0.05 was chosen as a threshold.

4.8 Functional studies by RNA interference (RNAi)

RNA interference is a powerful molecular research tool to suppress target gene expression for investigating the functional role. Double-stranded RNA are tailor-made to trigger the gene silencing. To inhibit gene expression, dsRNA was synthesized. Once in the cell, dsRNA recognized by dsRNA endonucleases (Type III RNAse) called Dicer. The enzyme dicer cleaves the dsRNA into small fragments of 21-23 nt of inhibitory RNAs (siRNAs). siRNAs integrate into RISC complex (RNA Induced Silencing Complex) and Slicer (RNAse H enzyme) removes the sense strand which results in the activation of RISC complex. Activated RISC binds to the target mRNA by base-pairing mechanism, causing mRNA cleavage. This prevents protein translation resulting in a gene silencing (Figure 4.1). RNAi was originally discovered in *Caenorhabditis elegans* (Fire et al., 1998) and has been studied in a variety of organisms including insects, protozoa and mammals for gene function analysis (Geldhof et al., 2007).

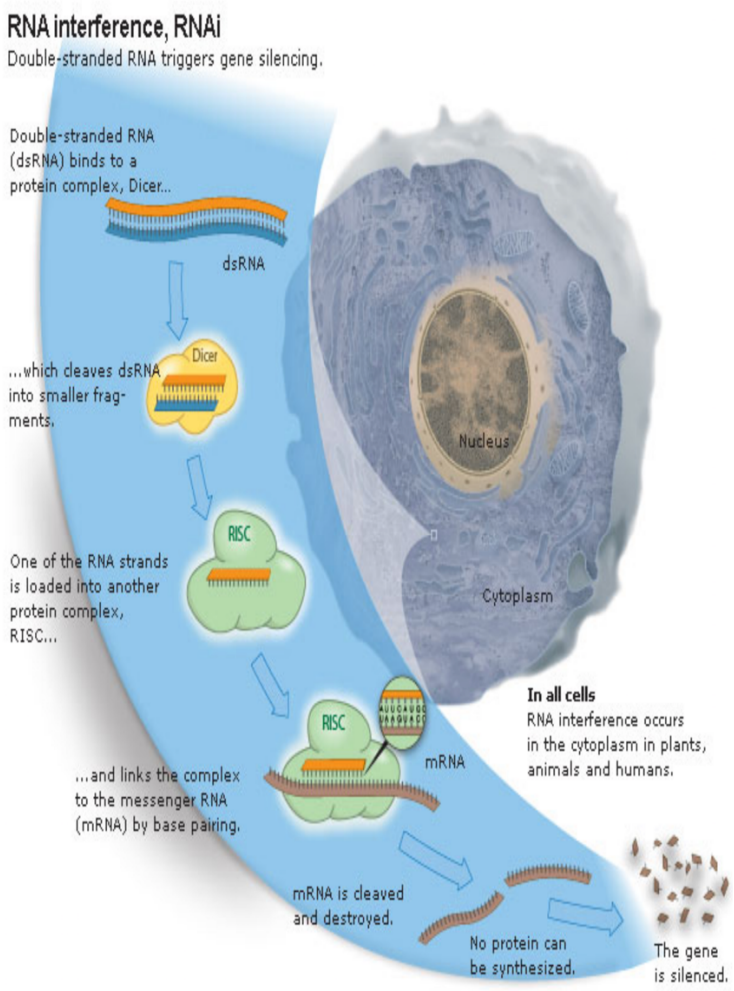


Figure 4.1: Schematic representation of the RNAi mechanism. When dsRNA enters the cell, the enzyme dicer binds to the dsRNA and cuts it into short pieces (SiRNA). Together with ribonucleoprotein particles (RNPs), SiRNA binds to the RISC complex, which then decomposes the mRNA and prevents translation. Figure is adapted from http://www.nobelprize.org.

4.8.1 Synthesis of double stranded RNA (dsRNA) for RNAi

dsRNA fragments were generated to knock down the target genes (LsDopamine1-2) for use in RNAi. Four primer pairs with T7 promoter were used to carry out two PCR reactions (Table 4.6). PCR products were verified on 1% agarose gel at correct sizes. In order to produce dsRNA from the above verified PCR products, the following procedure was followed as described in MEGAscript RNAi kit (Ambion). The protocol started with assembly of two high yield transcription reactions with overnight incubation at 37 °C. Annealing of RNA occurred with

incubation of the transcription reactions containing complementary RNA at 75 °C for 5 minutes. In order to anneal RNA, the transcription reactions containing complementary RNA were incubated at 75 °C for 5 minutes. RNA started to anneal as the reactants cooled. Formation of dsRNA occurred during the cooling down process. The next step involved the nuclease digestion for 1 hour incubation at 37 °C in order to remove DNA and single stranded RNA. The dsRNA was purified to remove proteins, nucleic acids and free nucleotides and eluted two times with elution buffer. The concentration of the purified dsRNA was measured by spectrophotometer (Nanodrop ND-1000, Thermo fisher Scientific).

4.8.2 RNAi; incubation of dsRNA in Nauplius I larvae

In order to get significant down regulation of the candidate genes, RNAi must be performed at the right time point (Eichner et al., 2014). During the molting from nauplii I to II, the larvae takes the dsRNA along with solution present in their surroundings.

Egg-string pairs were incubated in individual hatching wells and ~6-10 hours after hatching nauplius I larvae were subjected to dsRNA treatment (Figures 4.2 and 4.3a). 5-7 groups of approximately 35 nauplii in 150 µl sea water were transferred into the lids of eppendorf tubes distributed in a petri dish. 1.5 µg of dsRNA fragment was added and Petri dishes were placed carefully on a hatching board followed by incubation at 8.5 °C for ~10-12 hours. Along with treatment group, a parallel control group (a cod trypsin gene, CPY185 with no significant sequence similarity to the salmon louse examined by Dalvin et al. (2009) was used. Prior to transferring the samples into flow-through hatching wells, eppendorf lids were checked for exuvia (exoskeleton of sea lice) under microscope in order to confirm the naulius I had molted into the nauplius II stage (Figure 4.3b). After this, excessive dsRNA in solution were flushed with sea water, nauplius II larvae were incubated at 8.5 °C in a flow through system. All groups were examined under binocular microscope to observe any phenotypic changes. For each group (treatment and control CPY185) samples for assessment of knock-down were taken in the copepodid stage 7 days after exposure to dsRNA. Mortality rate was calculated for each group and samples were photographed and stored in 0.5 ml RNA later.

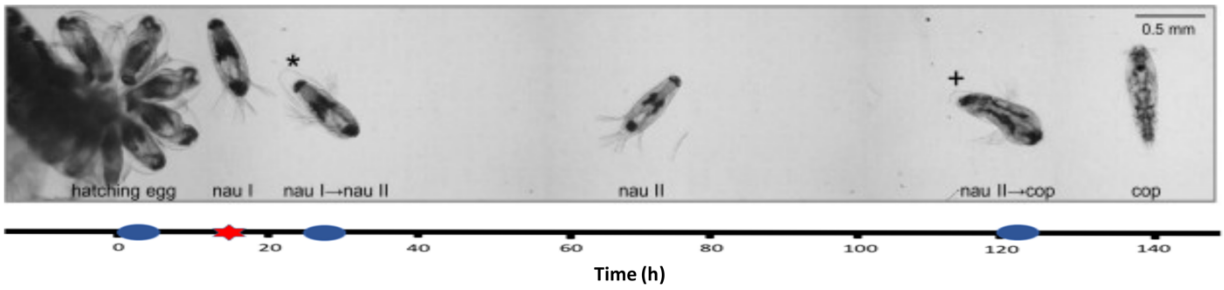


Figure 4.2: Time series of hatching and development of planktonic larval stages of *L. salmonis*. As the eggs hatches, nauplius I (nau I) is released. About after 6-17 hours, molting from nauplius I to nau II takes place followed by the shedding of exoskeleton (*). Nauplius II molts (+) into copepodids (cop) ~ 120 hours after hatching. Scale bar is 0.5 mm. Hatching time and molting is indicated by blue oval circles. RNAi treatment time is indicated by a red star. Soaking in dsRNA occurs in nau I stage , starting approximately 6-17 hours after hatching and lasts for 12~17 hours. Laboratory conditions: temperature 8.5 °C and salinity 34.5 ppt. Figure adapted and modified from C. Eichner et al. (2014).

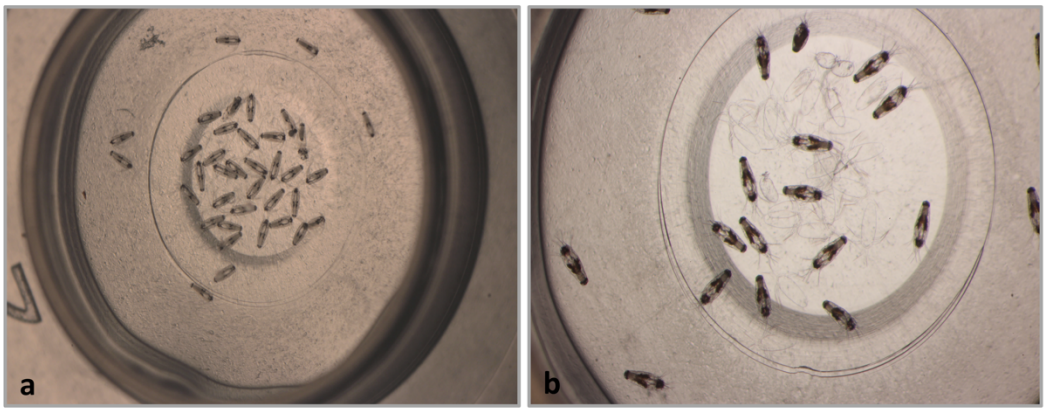


Figure 4.3: Exposure of larvae to dsRNA and the shedded exuvia

For LsDopamine1 two RNAi experiments were performed to confirm knock-down in expression levels and to infect fish. From the first RNAi experiment with LsDopamine2 the surviving copepodids larvae were pooled in two groups of 70 animals to confirm down regulation by qRT-PCR. The experiment was repeated to confirm the death of previously collected copepodids treated with LsDopamine2 and to measure down-regulation in the nauplius II stage before they start to die. Approximately 240 nauplii II were harvested from the control group and the experimental group two days post incubation during the repeat. Nauplii II were grouped in two parallel replicates (biological) for qRT-PCR analysis. Surviving copepodids were harvested from each group after 7 days post incubation. The knock-down experiment was repeated again with LsDopamine2 for obtaining optimum numbers of copepodids for five biological replicates.

During the repeat experiments of LsDopamine2 1 µg concentration of dsRNA was used with 100 µl nauplii solution.

4.8.3 Collection of lice samples for ontogenic analysis

Samples were taken four days after hatching (nauplius I-II), for nauplius I young (0-2 h), middle (10-12 h) and old (20-23 h), for nauplius II young (25-27 h), middle (74 h) and old (110 h), at day 7 (planktonic copepodids), (parasitic copepodids 2 days post infection), Chalimus I 9 days post infection, Chalimus II 15 days post infection. Samples for preadult I-II (male and female), unmature adult female and adult (male and female) were taken form RNA later in the defined stages by Christiane Eichner.

4.8.4 Infection with LsDopamine1 RNAi treated copepodids

4.8.4.1 Host and Experimental design

RNAi treated copepodids *L. salmonis* (infective stage) were used for infecting the salmon fish. A batch of Atlantic salmon (*Salmo salar*) was used for infection experiment. Before infection, water is lowered and flow rate was reduced to 120L/h and copepodids were placed carefully to the fish tank containing sea water with a salinity of 34.5 % and temperature of ±10 °C. Flow rate was set up to normal 360L/h later and dsRNA treated 100 copepodids were used $fish^{-1}$ for control and experimental group (Fig 4.4 A). Prior to infection, 100 copepodids from each group were separated from the RNAi experiment, which were further analyzed by qRT-PCR.

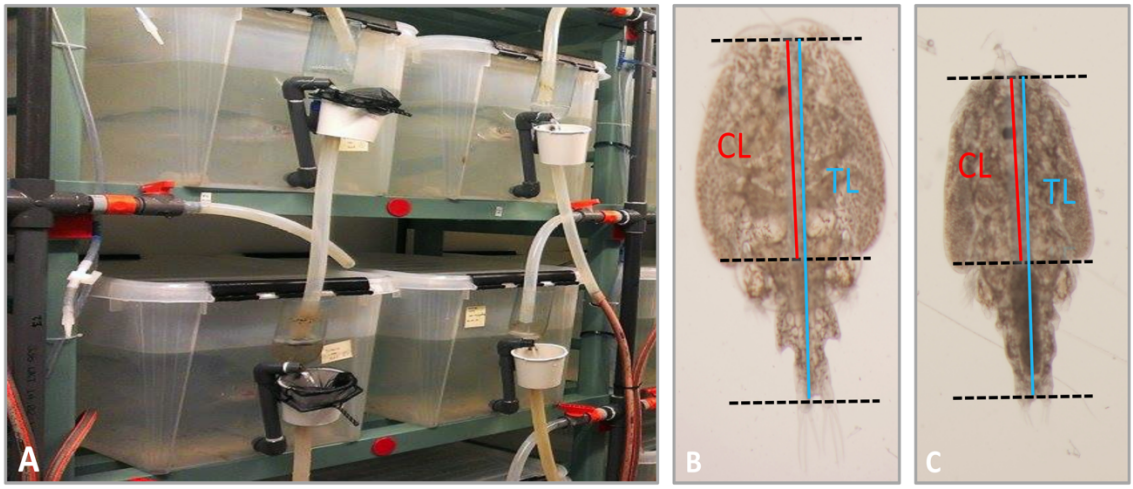


Figure 4.4: Experimental setup. (A) Control and experimental groups were divided into four tanks with single fish i.e. Control 1-II and LsDopamine1 I-II respectively. Lice were sampled 21 days post infection (DPI). Measurements were performed on pictures by calculating cephalothorax length (CL) and total length (TL). (B) Pre-adult I female. (C) Chalimus II.

4.8.4.2 Termination and Sampling

Atlantic salmon and copepodids were inspected twice in a week and the experiment was terminated 21 days post infection. The lice were then sampled from the fish by forceps from fish skin, gills, fins and mouth cavity. Prior to sampling, the fish were netted and killed by a sharp blow to the head in order to sample chalimus II lice properly. Chalimus II, pre-adult I male, pre-adult I female and pre-adult II male from each group were observed under microscope and photographed by placing the lice in a drop of seawater in a petri-dish and covering with a cover slip. The lice were then placed on RNA later (Ambion, Applied Biosystems) or 4% paraformaldehyde in PBS. Measurements were done on pictures, and Total Length (TL) and Cephalothorax Length (CL) was determined for each louse and the length was calculated by converting pixels into mm after photographing a scale (Figure 4.4B, C). Pictures were handled and measured in ImageJ v 1.49 software (National Institute of Health, USA). Numbers of recovered lice stage was determined for control (Control I-II) and experimental groups (LsDopamine1I-II) and CL/TL ratios of each louse was observed to distinguish the instar age. Moreover, maximum CL/TL, minimum CL/TL and mean CL/TL ratio was calculated. Comparable pre-adult I female lice were chosen from each group to confirm the knockdown of LsDopamine1 gene by qRT-PCR analysis.

4.9 *In Situ* Hybridization

In situ hybridization (ISH) is a technique used to precisely localize gene expression within a histologic section or whole mount by the hybridization of labelled complementary DNA or RNA to localize a known target DNA or RNA sequence within a tissue sample (Jin and Lloyd, 1997). In the present study, antisense probes were used to localize the LsDopamine1 and 2 mRNA in copepodids and preadult I female lice, using sense probes as a negative control. *In situ* hybridization was performed according to Kvamme et al. (2004) with some modifications to be specifically adapted for *L. salmonis* samples. List and compositions of all buffers used for ISH are given in Material section.

4.9.1 RNA probe synthesis from PCR products

For LsDopamine1 and LsDopamine2 sense and antisense probe synthesis, the primers with and without T7 promoter were used to produce sense and antisense DNA sequences (Table 4.6). Fragment size was verified by 1 % Agarose gel, and purified using UltraClean® 15 DNA Purification Kit (MO BIO) from PCR solutions. To synthesize probes, purified templates were incubated in a thermal cycler (Applied Biosystems Genemap PCR system 2700) at 37 °C for 2 hours using DIG RNA labeling kit in nuclease free conditions. For DNase treatment, probes were incubated at 37 °C for 15 minutes in DNase I, followed by inactivation by 0.2 M EDTA. Precipitated RNA pellet was washed with chilled 70 % ethanol and dissolved in DEPC water. Probe yield was determined by Nanodrop followed by spot test for verification.

For spot test preparations, three solutions were made: washing buffer A, 1 % blocking solution and detection buffer A (Section 3.1.1). In addition, a 1:2 dilution series of 5 was prepared followed by diluting standard control to (200 ng/µl) 1:200 and probes to 1:400. 1 µl of each dilution was placed on a positively charged nylon transfer membrane (Hydrabond N membrane), exposed to UV-light for 1 min and washed with 10 ml washing buffer for 20 seconds. Blocking was performed in 10 ml blocking solution A for 30 minutes by gently agitating to prevent unspecific binding followed by the addition of 2 antibodies (Anti-Dig- AP) to the blocking solution for 30 minutes incubation. The membrane was washed 3 times for 5 minutes with 10 ml

washing buffer A, followed by 1 minute washing with 10 ml detection buffer A. Detection was performed with 10 ml detection buffer A, 43 NBT and 35 BCIP and tube was wrapped with aluminum foil, followed by gentle agitation for 3-10 minutes. Finally, the membrane was washed with Milli-Q water, dried and photographed.

Table 4.6: Primers used for RNAi and *In situ* hybridization

Transcript	Primer	Sequences (5' - 3')
LsDopamine1	b3809forward	TCCCACATTTAAACGGGGCTATT
	b3486reverse_T7	TAATACGACTCACTATAGGGAGAATGTGGAGTCTGGTCCTCCC
	b3810reverse	ATGTGGAGTCTGGTCCTCCC
	b3485forward_T7	TAATACGACTCACTATAGGGAGATCCCACATTTAAACGGGGCTATT
LsDopamine2	b3811forward	TGCTCGAGAAAAGGTTCTGC
	b3730reverse_T7	TAATACGACTCACTATAGGGAGACCTTTTCCTGTAGCGTTGTTGA
	b3812reverse	CCTTTTCCTGTAGCGTTGTTGA
	b3729fprward_T7	TAATACGACTCACTATAGGGAGATGCTCGAGAAAAGGTTCTGC

4.9.2 Hybridization on paraffin slides

Preadult I female and copepodids *L. salmonis* were fixed on 4 % paraformaldehyde and sent to the Institute of Marine Research (IMR) for imbedding into the paraffin slides. Sectioning was done in LEICA RM 2155 at Department of Biology, University of Bergen.

Horizontal section of salmon lice (3 μm) were pretreated before ISH. The slides were baked for 20 minutes at 60 °C and paraffin was removed by washing 3 times for 10 minutes in 50 ml histoclear. Rehydration was performed by exposing the sections to decreasing concentrations of ethanol (100 %, 95 %, 70 % and 50 %) in DEPC water with 1-minute incubation after each rehydration in RNase free cuvettes. Finally, the slides were soaked twice in 2 X SSC buffer for 1 min. After rehydration, the slides were treated with proteinase K solution mix for 5 min in order for the tissue to open up for allowing probe entry. Tissue slides were fixed with 50 ml 4 % paraformaldehyde in 1xPBS to maintain the histological structure. Acetic anhydride treatment

was performed for 5 min that inactivates endogenous phosphatase followed by soaking in 2 X SSC buffer and dehydration step. A hydrofobe frame was made using RNase free PAP pen. Probe preparation was performed by boiling the probes with hybridization solution. The probes were then added to the slides and incubated overnight at 60 °C.

After overnight incubation, slides were gently flushed with 2 X SSC, and then washed twice with 50 ml 2 X SSC for 30 min at room temperature. The slides were then washed with 25 ml deionized formamide in 25 ml 2 X SSC for 30 min at 65 °C. Before RNase treatment, slides were washed twice for 10 minutes with 2 X SSC buffer at 37 °C. RNA digestion lasted for 30 min at 37 °C with 50 ml RNase buffer and 0,02 mg/ml RNase A, followed by 3 washing steps with 50 ml 1 x maleate buffer for 10 min at RT. For immunohistochemical detection, tissue slides were blocked with 45 ml 1 x maleate buffer, 0.05 % Triton X-100 and 1 % blocking solution, and then incubated for 2 hours at room temperature. Next, the tissue slides were washed twice for 5 minutes at room temperature with 50 ml 1 x maleate buffer. After the last washing step, slides were dried with whatman paper and 100 µl of Anti-Dig-AP FAB fragment was added to 1 ml blocking solution from the previous step. The tissue slides were placed overnight at RT in a moisture chamber.

The next day, tissue slides were washed two times with 50 ml 1 x maleate buffer for 10 minutes and one time with 50 ml processing buffer for 10 min. For processing, chromogen-substrate was prepared in 50 ml tube packed in aluminium foil with 22,5 µl NBT, 7 µl BCIP and 5 ml processing buffer. 200 µl solution was added to the color chamber and the slides were placed upside down as a lid. The incubation time ranged from a couple of hours to overnight depending upon the probe. The slides were placed in a stop buffer as soon as development was observed. Tissues were preserved by covering with some drops of with ImmunoHistoMount medium. Later, the tissues were covered with cover glass and left overnight to dry before sealed with nail-polish coat and stored at 4 °C. Finally, the images of slides were taken under the microscope.

4.10 Hematoxylin and erythrosine staining (HES)

HES staining is the most common technique to visualize tissues and anatomy of lice under microscope. HES staining was performed on copepodids and preadult I female lice to characterize the cell types by comparing with *in situ* hybridization slides. Hematoxylin stains the basophile parts of a cell in blue (the nucleus), while erythrosine stains the acidophile parts of cell in red (the cytoplasm) (Table 4.7).

Before performing the HE(S) staining the paraffin needs to be replaced with water through several infiltration baths. Prior to hydration, slides were incubated at 70 °C for 30 minutes and soaked in Histoclear twice for 10 minutes. Slides were washed with different concentrations of ethanol for 5 minutes each time. In the last step of hydration, slides were washed with tap water for 5 min. For mounting, the slides were dried and covered with histomount and a cover slip on.

Table 4.7: HE(S) staining steps

Solution	Time (minutes)
Hematoxylin	2,5
Tap water	4
1 % Erythrosin, pH=6,5	1,5
Tap water	1
96 % ethanol	1
100 % ethanol	1
100 % ethanol	1
Histoclear	5
Histoclear	5

5. Results

5.1 Sequence analysis and domain prediction of LsDopamine1-2

A total of eight genes were retrieved from LiceBase based on BLAST search with dopamine receptor sequences from other species against salmon louse genome and three genes i.e. EMLSAG00000003021, EMLSAG00000003269 and EMLSAT00000003268 were selected for further studies on the basis of their hits with dopamine receptor by BLAST search (NCBI). The predicted dopamine receptor from cDNA of *L. salmonis* EMLSAG00000003021 gene was characterized as LsDopmine1 and the length was verified by polymerase chain reaction (Figure 5.1). This cDNA was sequenced before by Aina-Cathrine Øvergård at SLRC.

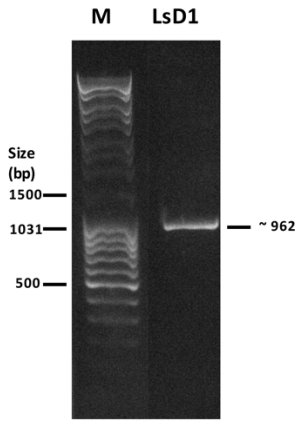


Figure 5.1: Amplification profile of predicted cDNA of LsDopamine1. The predicted dopamine receptor cDNA of LsDopamine1 (LsD1) is verified by polymerase chain reaction by using forward (b3464) and reverse (b3465) primers with an expected band length of 962 bp (lane 2). While lane 1 shows the marker.

The predicted dopamine receptor cDNAs of *L. salmonis* EMLSAG00000003269 and EMLSAG3268 were positioned next to each other on the same genomic supercontig, but as a stop codon was present in EMLSAG00000003269 sequence they were marked as two genes in LiceBase. NCBI BLAST search of EMLSAG00000003268 and EMLSAG00000003269 also displayed partial receptor sequences. To verify if EMLSAG00000003268 and EMLSAG00000003269 are parts of the same gene, the 5' RACE (Rapid Amplification of cDNA ends) and 3' RACE were performed and cDNA products were cloned and sequenced. Gene

specific primers were used to obtain full length cDNA sequence of the (EMLSAG00000003269+ EMLSAT00000003268) receptor gene. Sequence analysis revealed this to be the LsDopamine2 sequence along with two splice variants which were identical in the 5' end and had distinct sequence at their 3' end (Figure 5.2). Sequencing also revealed an exon 6 which was not predicted in LsDopamine2 cDNA sequence in LiceBase. The 5' and 3' RACE products overlap and link the two predicted genes in LiceBase and the length of new transcript (LsDopamine2) was 1596 bp.

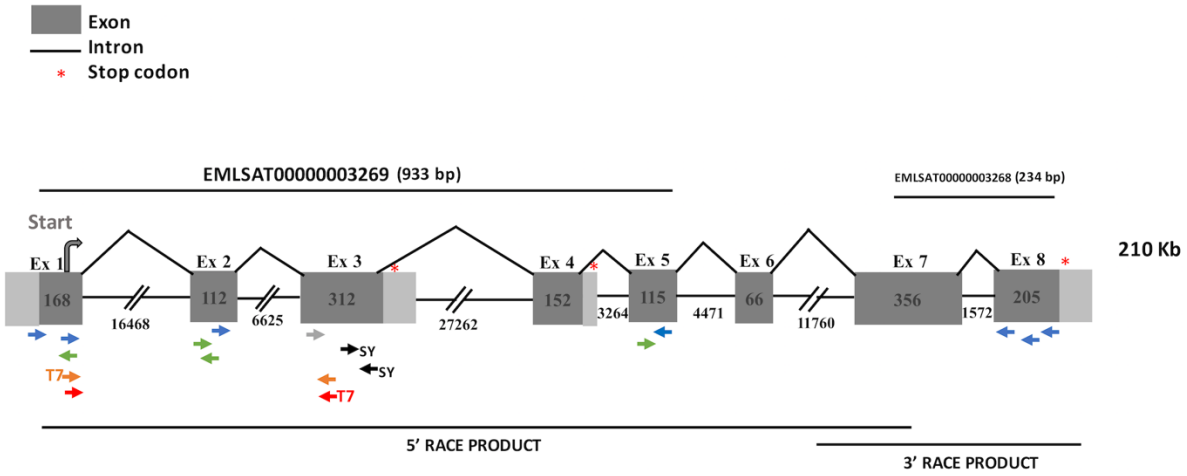


Figure 5.2: Schematic representation of exon-intron pattern of the *L. salmonis* dopamine D2-like (LsDopamine2) receptor on the genomic sequence. Cloning and sequencing confirmed the presence of two different mRNA variations. The exons are shown by dark grey boxes (Ex 1-8), which were mapped to the genomic DNA of LsDopamine2 and the genomic sequence extend up to 210 kb. The introns are depicted as lines between exons with lengths in the number of nucleotides, while stop codons are shown by asterisk and translation start site is indicated by an arrow. Light grey boxes shows the extended poly A tail in the 3' end and bases before the first exon and also the extending splice variants in introns. Lines above the LsDopamine2 structure shows the EMLSAG00000003269 and EMLSAG00000003268 cDNA sequences with length retrieved from LiceBase. Blue dotted lines indicate the position of exons on LiceBase retrieved cDNA sequences before sequencing. Lines below the LsDopamine2 structure represents the overlapping RACE products. General primers used for sequencing and PCR are shown by green, grey and blue arrows respectively. While, the primers used for probe synthesis for *in situ* hybridization and RNAi study are indicated by orange and red arrows. Primers used for SYBR green assay are highlighted with black arrows. Two mRNA variants are depicted as connecting boxes with exons and introns.

Variant 1 consisted of only one exon (Ex 3) and extended into the 3rd intron, similarly, the variant 2 started at exon 3 extending into exon 4 and ends into intron 4 (light grey box, Figure 2). Each intron junction followed the GT ..AG rule in the splice site. The cDNA sequences of the verified LsDopamine1 and LsDopamine2 were found to be 354 and 450 amino acids respectively. Furthermore, NCBI blast search, domain and protein structure prediction showed that LsDopamine1 and LsDopamine2 are members of the 7 transmembrane rhodopsin-like G protein-coupled receptors (GPCRs) (Figure 5.3). Alignment was made using Clustal and sequence similarities were found between LsDopamine1 and LsDopamine2 and with other arthropods (Figure 5.4A, B, C). As mentioned before, dopamine receptors have seven transmembrane domain structure with conserved amino acid residues, aspartic acid (D), serine serine (SS) and phenylalanine (F) in TM domain III, TM domain V and TM domain VI respectively (Missale, 1998).

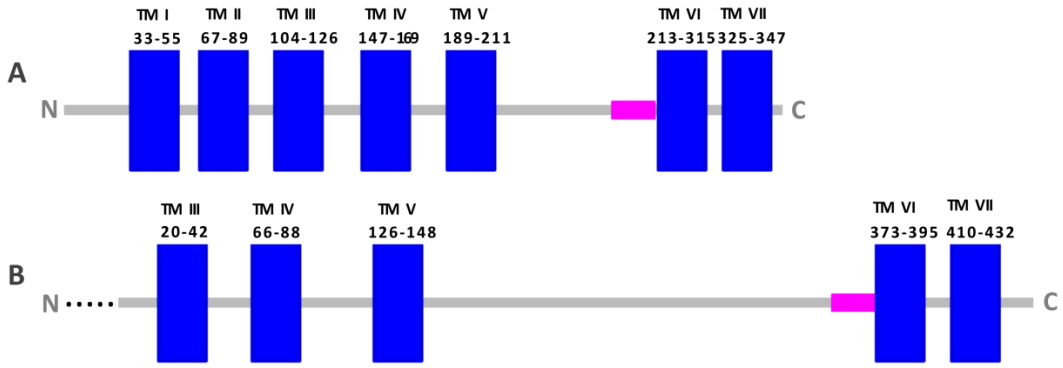
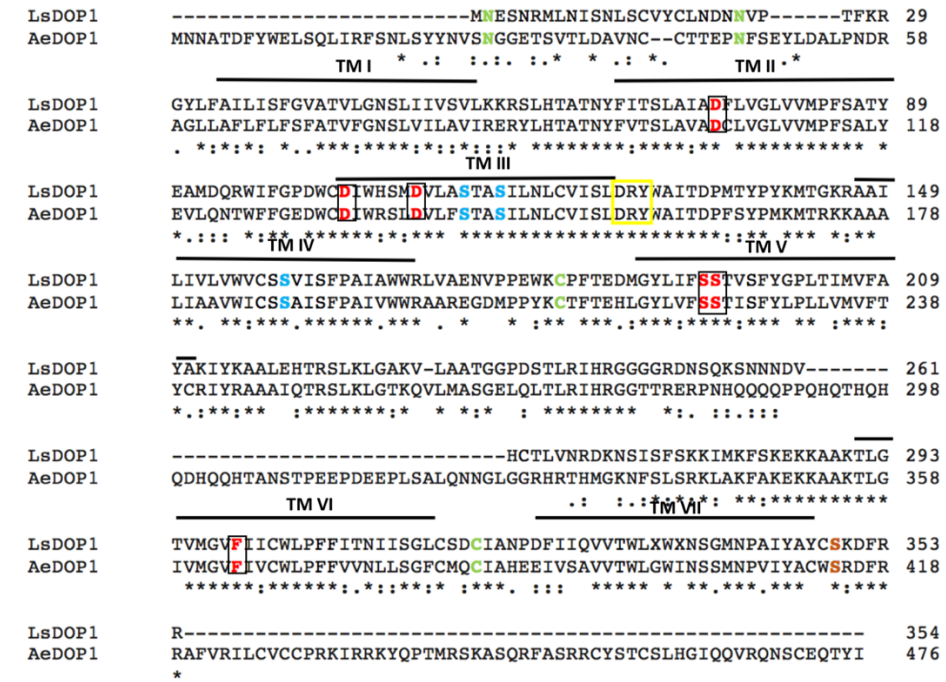
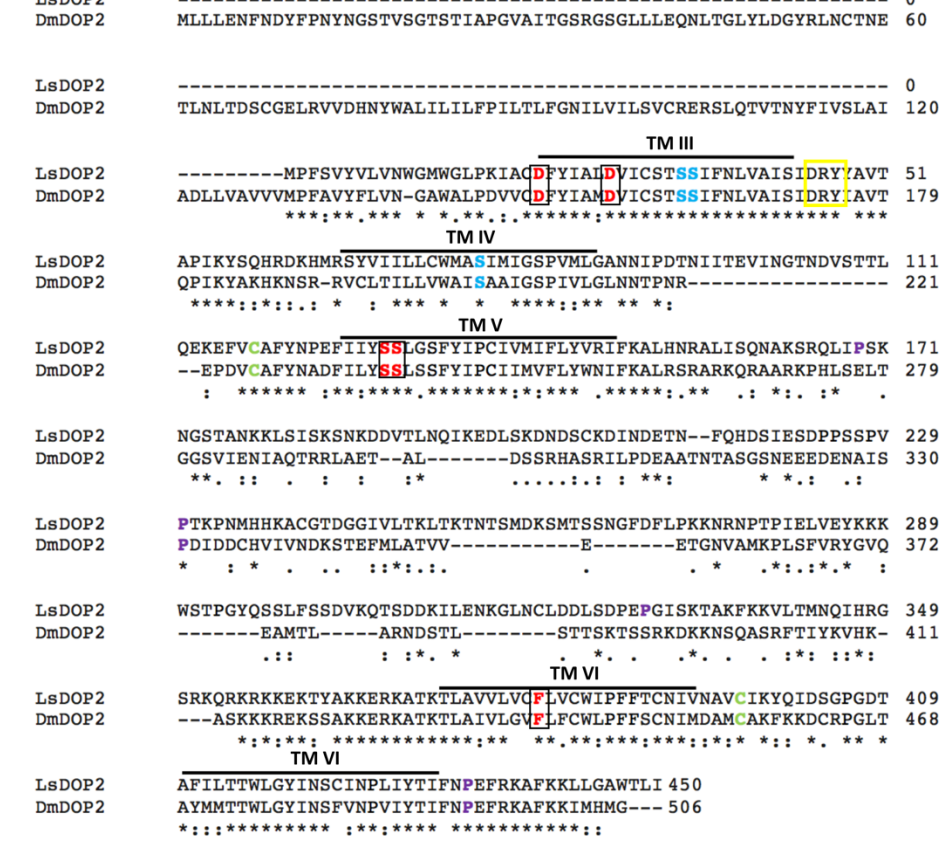


Figure 5.3: Domain structure of LsDopamine1 and 2. (A) LsDopamine1 354 amino acid long sequence consists of seven transmembrane domains (blue boxes) and small pink box represents low complexity region. (B) LsDopmaine2 450 amino acid long sequence derived from sequence analysis displayed five transmembrane domains represented by blue boxes and small pink box shows low complexity region. TM domains start and end positions are represented with numbers on top.

A


LsDOP1 ----------MNESNRMLNISNLSCVYCLNDNNVP------TFKR 29
AeDOP1 MNNATDFYWELSQLIRFSNLSYYNVSNGGETSVTLDAVNC--CTTEPNFSEYLDALPNDR 58
TM I
TM II
LsDOP1 GYLFAILISFGVATVLGNSLIIVSVLKKRSLHTATNYFITSLAIADFLVGLVVMPFSATY 89
AeDOP1 AGLLAFLFLFSFATVFGNSLVILAVIRERYLHTATNYFVTSLAVADCLVGLVVMPFSALY 118
TM III
LsDOP1 EAMDQRWIFGPDWCDIWHSMDVLASTASILNLCVISIDRYWAITDPMTYPYKMTGKRAAI 149
AeDOP1 EVLQNTWFFGEDWCDIWRSLDVLFSTASILNLCVISIDRYWAITDPFSYPMKMTRKKAAA 178
TM IV
TM V
LsDOP1 LIVLVWVCSSVISFPAIAWWRLVAENVPPEWKCPFTEDMGYLIFSSTVSFYGPLTIMVFA 209
AeDOP1 LIAAVWICSSAISFPAIVWWRAAREGDMPPYKCTFTEHLGYLVFSSTISFYLPLLVMVFT 238
LsDOP1 YAKIYKAALEHTRSLKLGAKV-LAATGGPDSTLRIHRGGGGRDNSQKSNNNDV------- 261
AeDOP1 YCRIYRAAAIQTRSLKLGTKQVLMASGELQLTLRIHRGGTTRERPNHQQQQPPQHQTHQH 298
LsDOP1 ----------HCTLVNRDKNSISFSKKIMKFSKEKKAAKTLG 293
AeDOP1 QDHQQHTANSTPEEPDEEPLSALQNNGLGGRHRTHMGKNFSLSRKLAKFAKEKKAAKTLG 358
TM VI
TM VII
LsDOP1 TVMGVFIICWLPFFITNIISGLCSDCIANPDFIIQVVTWLXWXNSGMNPAIYAYCSKDFR 353
AeDOP1 IVMGVFIVCWLPFFVVNLLSGFCMQCIAHEEIVSAVVTWLGWINSSMNPVIYACWSRDFR 418
LsDOP1 R---------- 354
AeDOP1 RAFVRILCVCCPRKIRRKYQPTMRSKASQRFASRRCYSTCSLHGIQQVRQNSCEQTYI 476

B


LsDOP2 ---------- 0
DmDOP2 MLLLENFNDYFPNYNGSTVSGTSTIAPGVAITGSRGSGLLLEQNLTGLYLDGYRLNCTNE 60
LsDOP2 ---------- 0
DmDOP2 TLNLTDSCGELRVVDHNYWALILILFPILTLFGNILVILSVCRERSLQTVTNYFIVSLAI 120
TM III
LsDOP2 ---------MPFSVYVLVNWGMWGLPKIACDFYIAIDVICSTSSIFNLVAISIDRYVAVT 51
DmDOP2 ADLLVAVVVMPFAVYFLVN-GAWALPDVVCDFYIAMDVICSTSSIFNLVAISIDRYIAVT 179
TM IV
LsDOP2 APIKYSQHRDKHMRSYVIILLCWMASIMIGSPVMLGANNIPDTNIITEVINGTNDVSTTL 111
DmDOP2 QPIKYAKHKNSR-RVCLTILLVWAISAAIGSPIVLGLNNTPNR---------- 221
TM V
LsDOP2 QEKEFVCAFYNPEFIIYSSLGSFYIPCIVMIFLYVRIFKALHNRALISQNAKSRQLIPSK 171
DmDOP2 --EPDVCAFYNADFILYSSLSSFYIPCIIMVFLYWNIFKALRSRARKQRAARKPHLSELT 279
LsDOP2 NGSTANKKLSISKSNKDDVTLNQIKEDLSKDNDSCKDINDETN--FQHDSIESDPPSSPV 229
DmDOP2 GGSVIENIAQTRRLAET--AL-------DSSRHASRILPDEAATNTASGSNEEEDENAIS 330
LsDOP2 PTKPNMHHKACGTDGGIVLTKLTKTNTSMDKSMTSSNGFDFLPKKNRNPTPIELVEYKKK 289
DmDOP2 PDIDDCHVIVNDKSTEFMLATVV----------E-------ETGNVAMKPLSFVRYGVQ 372
LsDOP2 WSTPGYQSSLFSSDVKQTSDDKILENKGLNCLDDLSDPEPGISKTAKFKKVLTMNQIHRG 349
DmDOP2 -------EAMTL-----ARNDSTL--------STTSKTSSRKDKKNSQASRFTIYKVHK- 411
TM VI
LsDOP2 SRKQRKRKKEKTYAKKERKATKTLAVVLVCFLVCWIPFFTCNIVNAVCIKYQIDSGPGDT 409
DmDOP2 ---ASKKKREKSSAKKERKATKTLAIVLGVFLFCWLPFFSCNIMDAMCAKFKKDCRPGLT 468
TM VI
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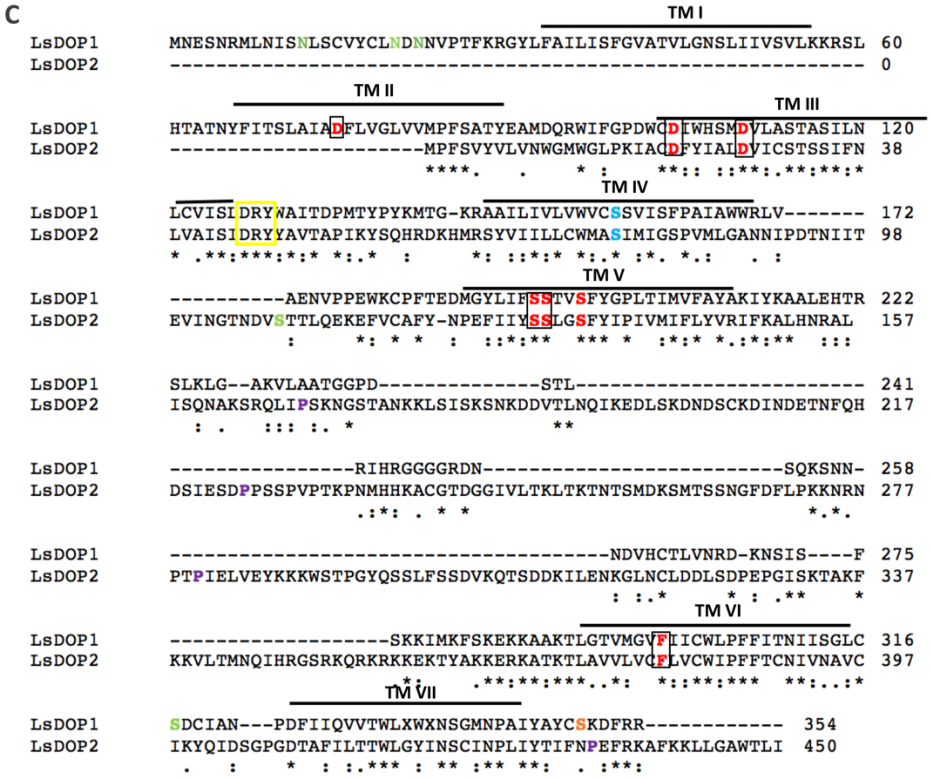

Figure 5.4: Alignment of LsDopamine1 (LsDOP1) and LsDopamine2 (LsDOP2) proteins with dopamine receptors from other species. (A) Alignment of LsDopamine1 (LsDOP1) with *Aedes aegypti* (AeDOP1, accession number: AFB73767) shows 57.2 % identity and 76.8 % similarities. (B) Alignment of LsDopamine2 (LsDOP2) with *Drosophila melanogaster* (DmDOP2, accession number: Q8IS44) shows 41.7 % identity and 63.1 % similarities. (C) Alignment of LsDopmaine1 with LsDopamine2 shows 30.0 % identity and 55.5 % similar sequences. Conserved amino acid residues and conservative changes are indicated by asterisk and colon respectively. Residue numbers are listed on the right. Seven transmembrane domains (TM I-VII) are indicated by lines over the residues for LsDopamine1. While, first two TM domains are missing in LsDopamine2. Possible glycosylation sites are highlighted in green color (A, C). Conserved residues (involved in dopamine binding) in TM III (Asp; D), V (Ser Ser; SS) and VI (Phe; F) are highlighted in boxes (A, B, C). (A) Conserved "DRY" motif in the cytoplasmic region of TM III is shown with yellow box (A, B, C). LsDopmaine1 show a long C- terminal with conserved serine (S) residue highlighted in orange and short 3rd intracellular loop (I3) lies between TM V-VI whereas, LsDopamine II displays a short COOH terminus and long I3 (B). Two cysteine residues are highlighted in green in extracellular loops 2 (TM IV-V) and 3 (TM VI-VII) involved in forming a disulphide bond. Serine residues in TM III-IV are shown in blue. (B) Residues that are targets for phosphorylation are highlighted in purple.

5.2 Phylogeny of LsDopamine1 (LsDOP1) and LsDopamine2 (LsDOP2)

Multiple sequence alignment was done using Clustal X and the phylogenetic tree is as shown in Figure 5.5. The phylogenetic tree was constructed using the amino acid sequences of octopamine, serotonin and dopamine receptors from a range of species belonging to both vertebrate and invertebrate classes. The Neighbour-Joining (N-J) plot construction method was used to determine the genetic relatedness of LsDOP1 and LsDOP2 sequences to similar proteins belonging to other classes of organisms as described below. The phylogenetic tree points out a similar grouping of the LsDOP1 sequence with similar proteins belonging to other arthropods i.e. AeDOP1 of *Aedes aegypti* and AmDOP2 of *Apis mellifera* with bootstrap values near 1000 for basic iterations of 111 and 1000. Similarly, the LsDOP2 sequence from *L. salmonis* seems to be evolutionarily related to crustacean and arthropods *Panulirus interruptus* and *Drosophila melanogaster's* D2-like dopamine receptors respectively also with a bootstrap value of around 1000 revealing a strong support for the phylogenetic tree construction.

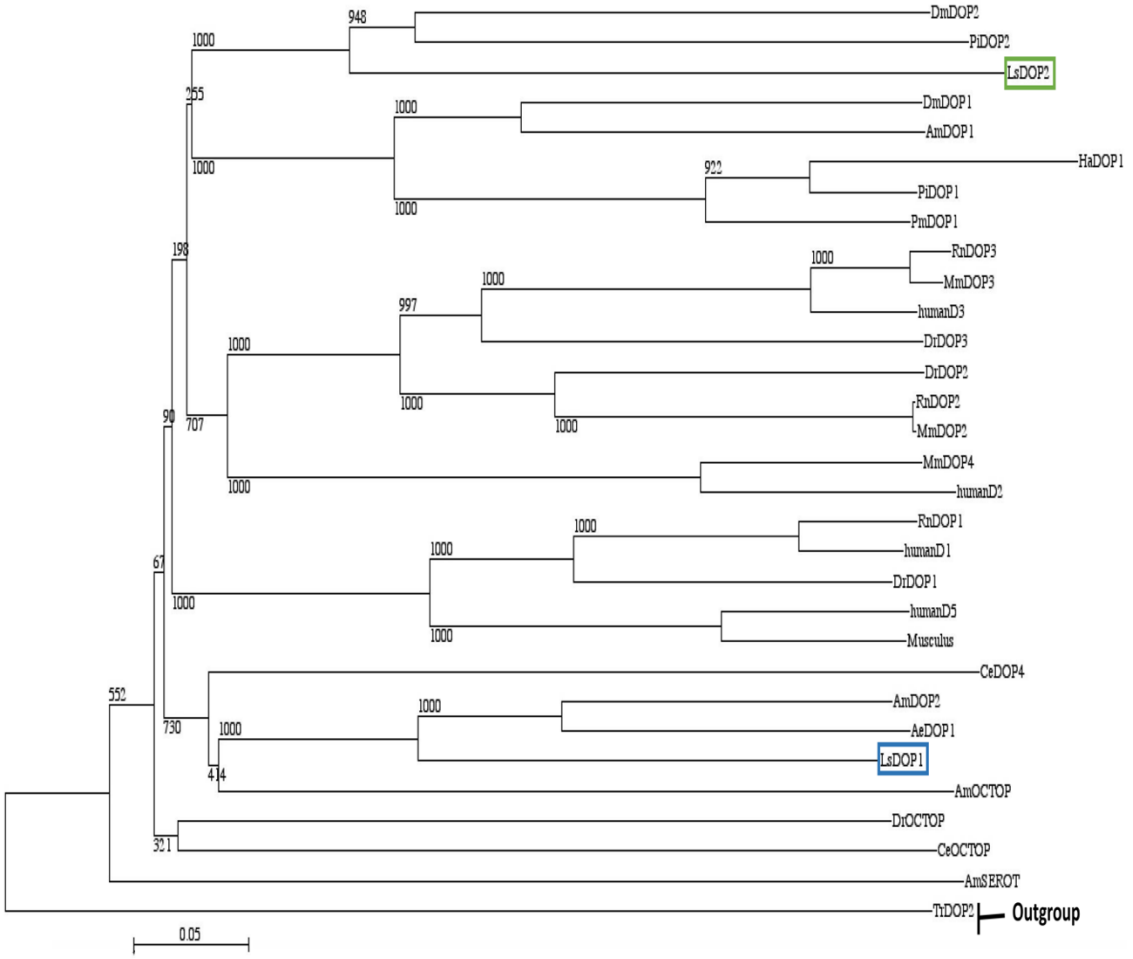

Figure 5.5: A phylogenetic tree with bootstrap values. Sequences from 24 previously reported dopamine receptors, three octopamine receptor, one serotonin receptor, one D2-like dopamine receptor (outgroup) and LsDOP1 and LsDOP2 (highlighted blue and green) were used to create a phylogenetic tree. The scale bar with number 0.05 represents a branch length is proportional to the amount of genetic change. Sequences: DmDOP2, Drosophila dopamine receptor, accession number: Q8IS44; PiDOP2, Panulirus interruptus dopamine receptor: ABI64137; DmDOp1, Drosophila dopamine receptor: AAA85716; AmDOP1, Apis mellifera dopamine receptor: NP_001011595; HaDOP1, Homarus dopamine receptor: ADA68262; PiDOP1, Panulirus dopamine receptor: ABB87183; PmDOP1, Penaeus monodon dopamine receptor: AFX71574; RnDOp3, Rattus norvegicus dopamine receptor: AAA41076; MmDOP3, Musculus dopamine receptor: NP_031903; humanD3, human dopamine receptor: AAI28124; DrDOP3, Danio rerio dopamine receptor: NP_898890; DrDOP2, Danio rerio dopamine receptor: NP_898891; RnDOP2, Rattus norvegicus dopamine receptor: NP_036679; MmDOP2, Musculus dopamine receptor: NP_034207; MmDOp4, Musculus dopamine receptor: NP_031904; humanD2, human dopamine receptor: P21917; RnDOP1, Rattus norvegicus dopamine receptor: AAA70428; humanD1, human dopamine receptor: CAA41734; DrDOP1, Danio rerio dopamine receptor: ACI42369; hunamnD5, human dopamine receptor: CAA41360; Musculus dopamine receptor: EDL37553; CeDOP4, C. elegans Dopamine receptor: Q18775; AmDOP2, Apis mellifera dopamine receptor: XP_006561568; AeDOP1, Aedes aegypti dopamine receptor: AFB73767; AmOCTOP, Apis mellifera octopamine receptor: NP_001011565; DrOCTOP, Drosophila octopamine receptor: AAA28731; CeOCTOP, C. elegans octopamine receptor: CCD83473; AmSEROT, Apis mellifera serotonin receptor: CBX90120; TrDOP2, Takifugu rubripes dopamine receptor: XP_003972057.

5.3 Expression study of LsDopamine1 and LsDopamine2 transcripts

In situ hybridization was performed in *L. salmonis* life stages of copepodids and preadult I female lice in order to study the expression pattern using the LsDopamine1 and LsDopamine2 specific antisense probes and sense probes as a control. In general, unspecific staining of the outer cuticle layer was observed both for the sense and anti-sense probes which is common to many previous *in situ* hybridization experiments in sections of *L. salmonis* (pers comm Eichner, 2016). Expression of LsDopamine1-2 transcript was observed in the subcuticular tissues in the copepodids (Figures 5.6 and 5.8). In preadult I female, a relatively strong signal was detected in tegumental glands type I (pers. comm. A Øvergårdfor, 2016) for LsDopamine1-2 transcript expression (Figures 5.7 and 5.9). Haematoxylin and erythrosine stained preadult I female and copepodids were used for proper identification of cell types in *in situ* hybridization lice.

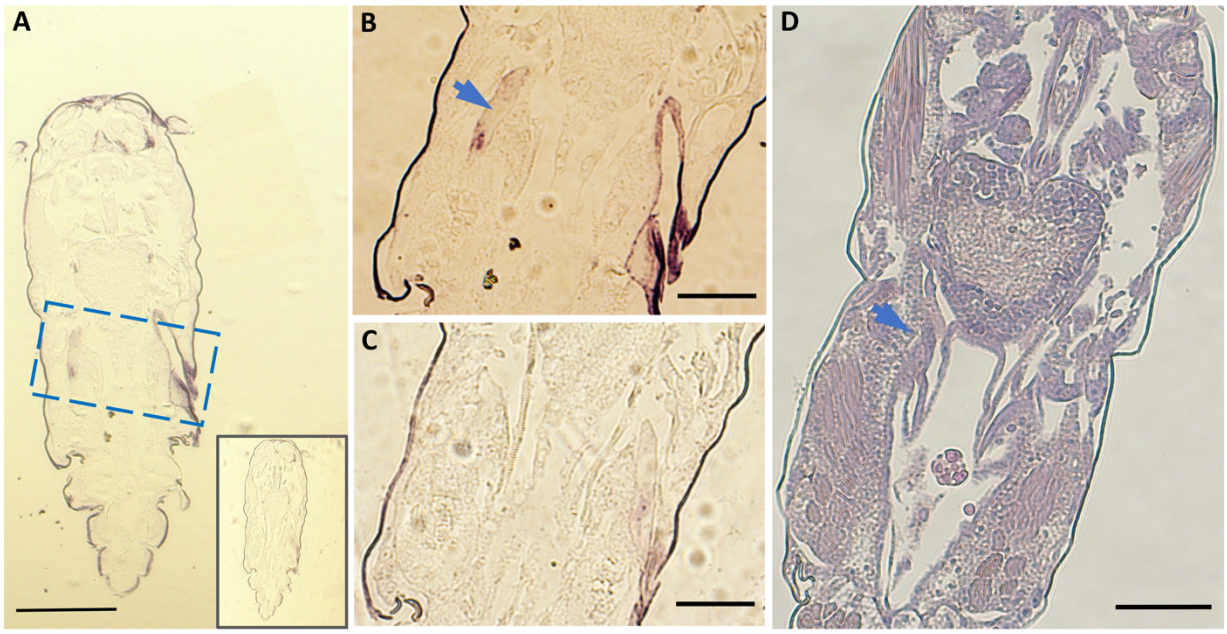

Figure 5.6: LsDopamine1 transcript localisation in *Lepeophtheirus salmonis* copepodids. The dashed square points out the copepodid's part (A) enlarged in picture B and C. Hybridization with sense probe is shown within a box (A). In situ hybridization is performed using LsDopamine1 specific antisense probe (B) and LsDopamine1 sense probe as a control (C). HES staining on whole section of copepodids is shown with an arrow (D). Blue arrow indicates the staining of LsDopamine1 receptor gene in subcuticular tissues in copepodids (B). Scale bar = 100 µm (A), 20 µm (B and C) and 50 µm (D).

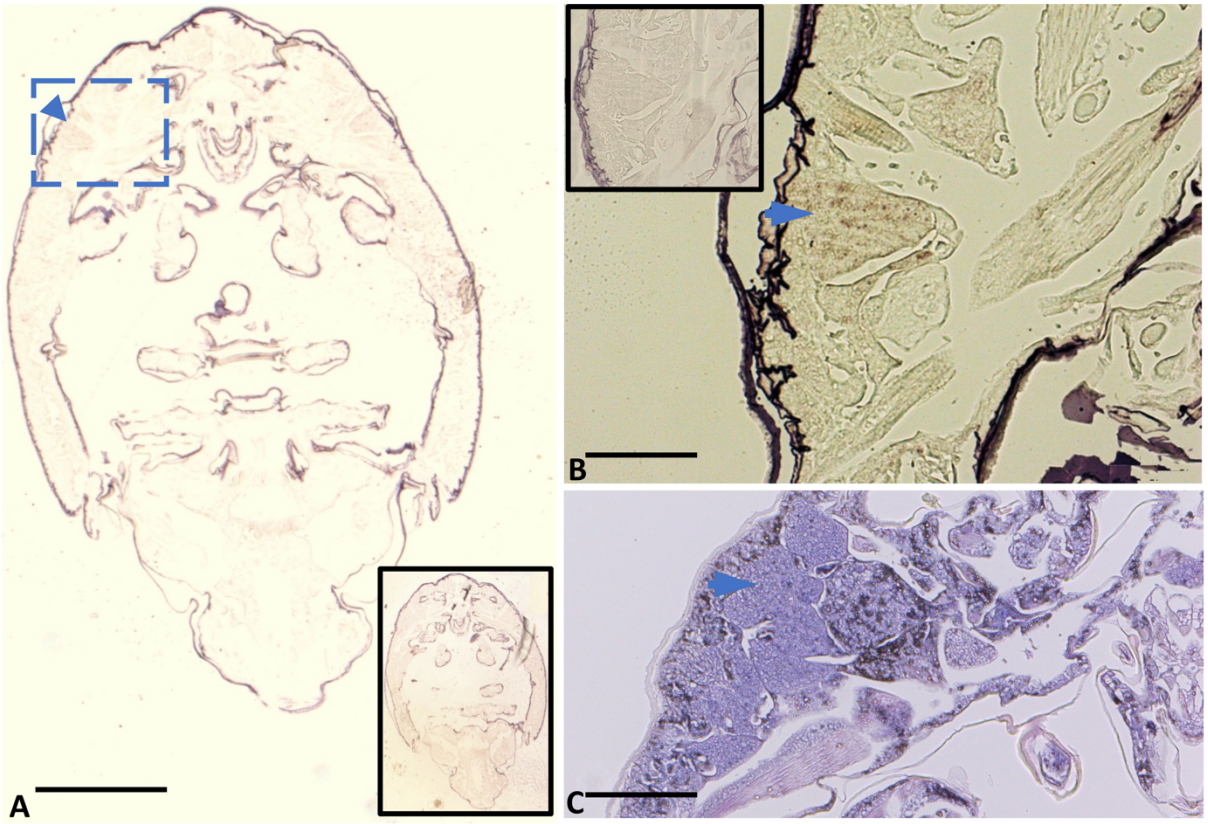

Figure 5.7: LsDopamine1 transcript localisation in *Lepeophtheirus salmonis* preadult I female. Arrow inside the dashed square in picture A (hybridization with anti-sense probe) shows LsDopamine1 expression in the glands (tegumental type 1) in anterior part of preadult I female enlarged in picture B, sense probe hybridization is shown in a small inserted picture B, where no signal was detected. HES stained glands are indicated by arrow (C). Scale bars indicate 500 µm (A), 50 µm (B and C).

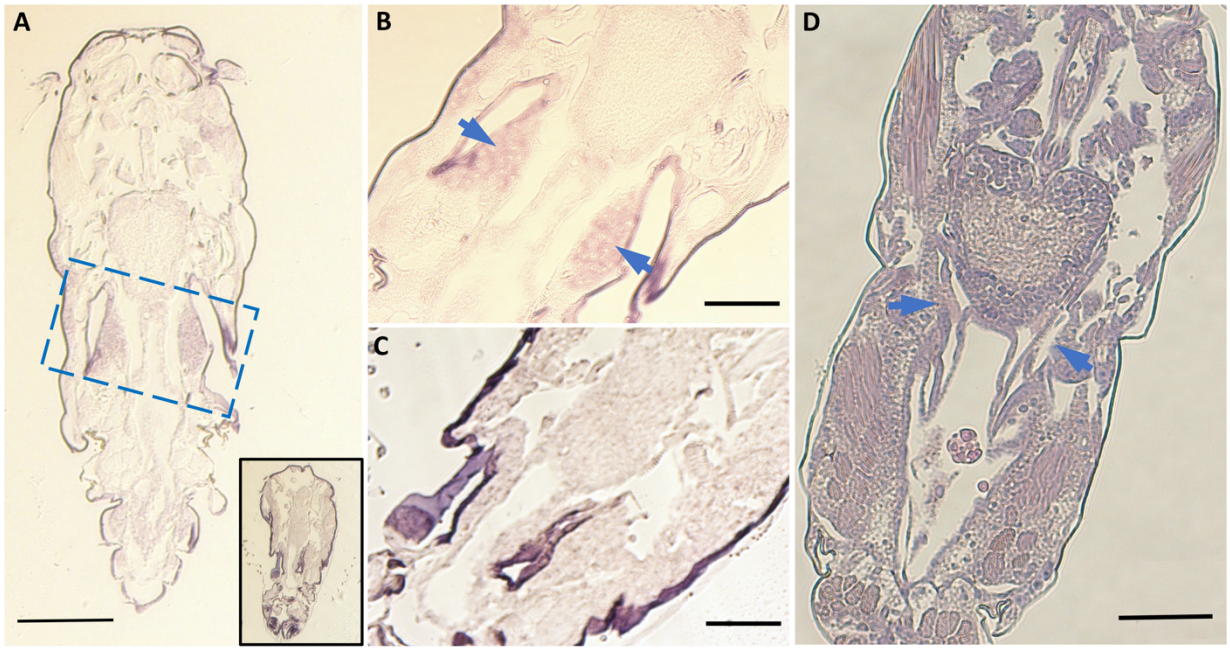

Figure 5.8: LsDopamine2 transcript expression in *Lepeophtheirus salmonis* copepodids. Strong hybridization of LsDOP2 receptor gene was detected in the subcuticular tissues of copepodids indicated by arrows (B) as compared to hynridization with sense probe (C). Picture (D) show neighbouring slide stained with hematoxylin and erythrosin. The dashed arrow indicates the position where the copepodid's tissue part is enlarged in picture B. Scale bar = 100 µm (A), 20 µm (B and C) and 50 µm (D).

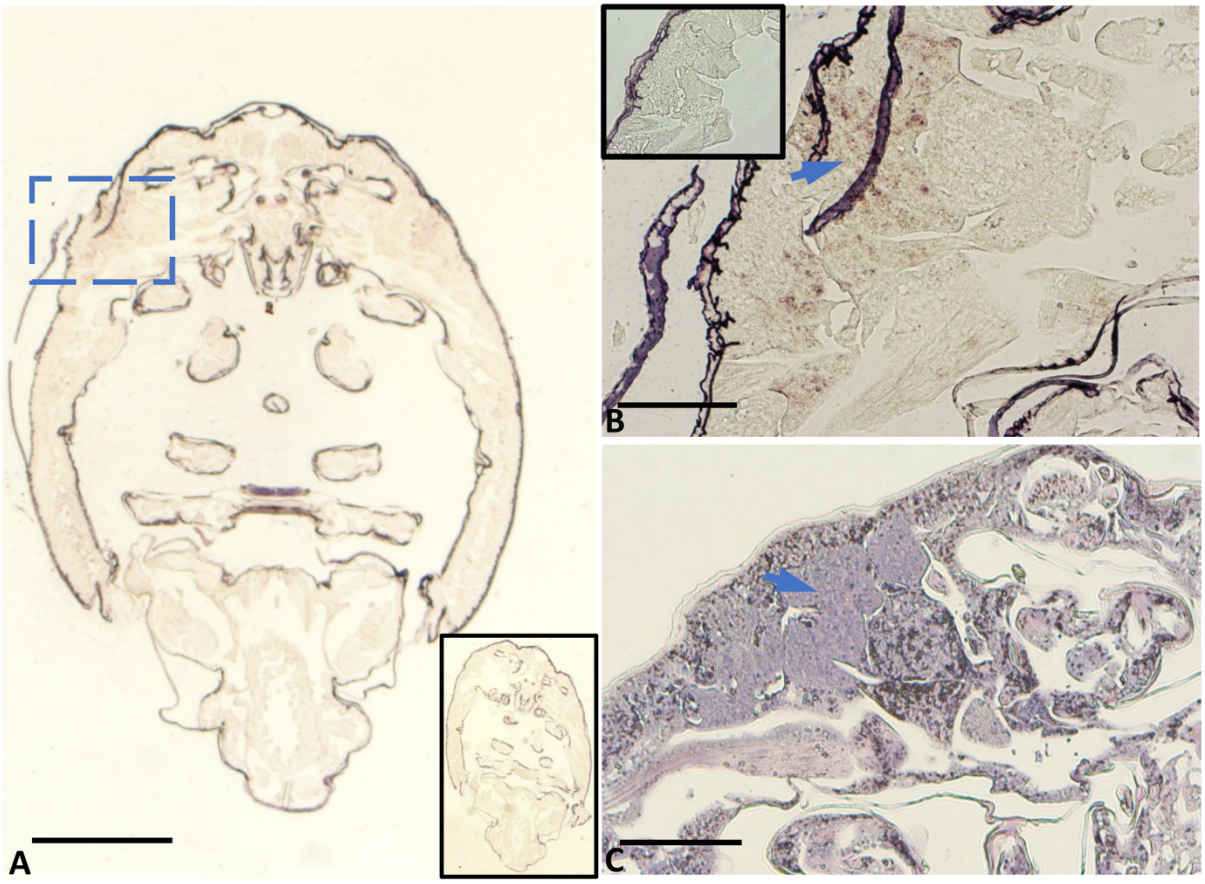

Figure 5.9: LsDopamine2 transcript expression in *Lepeophtheirus salmonis* preadult I female. Hybridized section exposed to LsDopamine2 antisense probe (A). B is a magnified view of antisense probe where arrow strongly indicates the expression of LsDopamine2 fragment in tegumental glands type 1 in the anterior part of preadult I female confirmed by HES stained section (C, indicated with arrow). No hybridization was seen in sense probe indicated as small box (B). Scale bars indicate 500 µm (A), 50 µm (B) and 100 µm (C).

5.4 Expression of LsDopamine1-2 at different developmental stages of lice

An ontogenic expression analysis was performed on LsDopamine1-2 receptor genes of *L. salmonis.* The expression levels of nauplius I to nauplius II were measured in young, middle and old stages to measure the variation in expression levels before or after molting. LsDopamine1 was observed to be at the highest expression levels in the adult male stage followed by nauplii II (middle) and planktonic and parasitic copepodids. The expression level varied a bit in rest of the stages, except for the nauplii II old that showed a bit higher expression level (Figure 5.10). Whereas, there were no major changes observed in LsDopamine2 expression levels during the

different developmental stages from nauplius I (young, middle, old) to II (young, middle, old) and very low levels of expression were found in chalimus I and II, preadult I and II male and female, unmature adult female and adult male and female. Higher expression levels were observed in copepodids (planktonic and parasitic) (Figure 5.11). This analysis showed that the expression levels for LsDopamine1 and 2 were up and down regulated as the organism progressed through its life cycle.

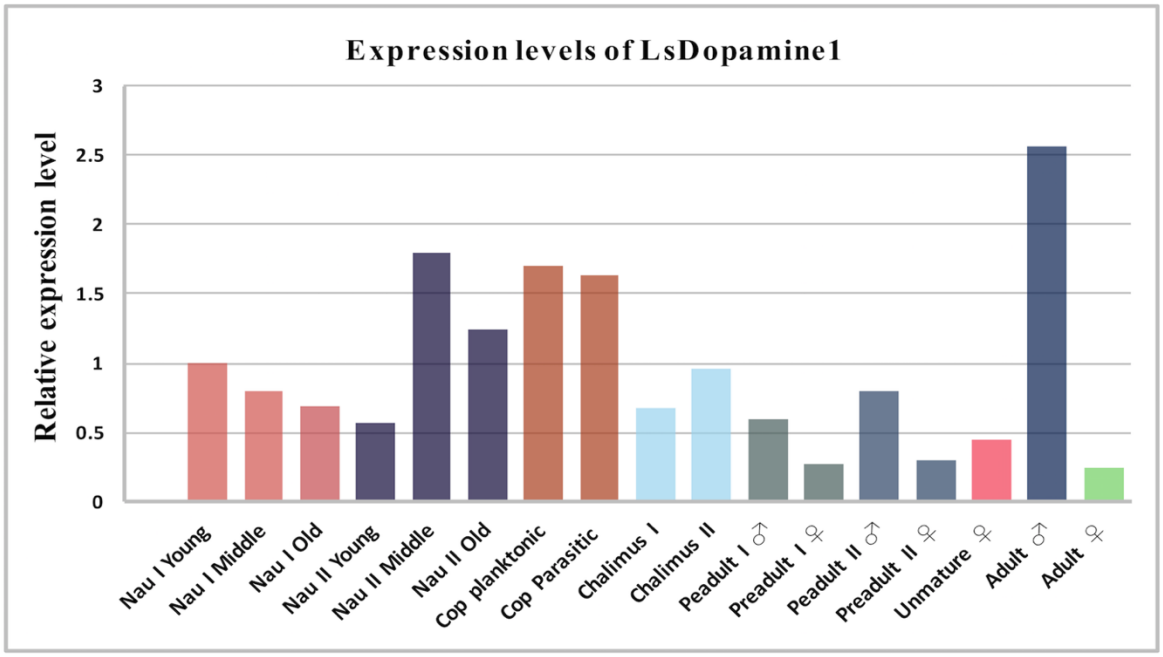

Figure 5.10: qRT-PCR analysis of expression pattern of LsDopamine1 in different developmental stages of L. salmonis. Mean values are normalized to eEF1α, and nauplii I young is set as a calibrator. Nau (nauplius), Cop (copepodids). Samples were taken four days after hatching (nauplius I-II), at day 7 (planktonic copepodids), (parasitic copepodids 2 days post infection), Chalimus I 9 days post infection, Chalimus II 15 days post infection. Whereas, samples for preadult I-II (male and female), unmature adult female and adult (male and female) were taken in the defined stages. LsDopamine I has high expression in adult male, nauplius II (middle) and copepodids (planktonic and parasitic), while it is up and down regulated in the remaining stages. Standard deviation varies from 0.1 to 0.7 with an average of 0.3. (n= 3).

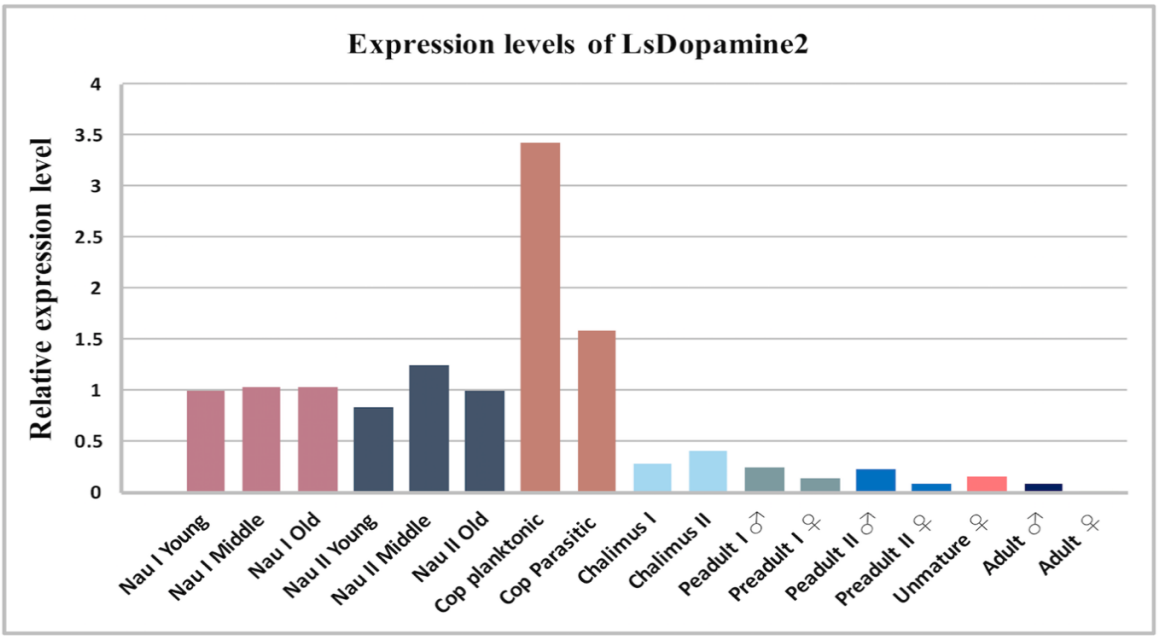

Figure 5.11: qRT-PCR analysis of expression pattern of LsDopamine2 in different developmental stages of *L. salmonis*. Mean values are normalized to eEF1α, and nauplii I young is set as a calibrator. Nau (nauplius), Cop (copepodids). Samples were taken two days after hatching (nauplius I-II), at day 7 (planktonic copepodids), (parasitic copepodids 2 days post infection), Chalimus I 9 days post infection, Chalimus II 15 days post infection. Whereas, samples for preadult I-II (male and female), unmature adult female and adult (male and female) were taken in the defined stages. LsDopamine II shows the highest expression in planktonic copepodids, followed by parasitic copepodids and naulpii I-II, whereas it is very low in the remaining stages. Standard deviation varies from 0.01 to 0.9 with an average of 0.4. (n= 3)

5.5 RNA interference studies

5.5.1 Functional assessment of LsDopamine1-2 by RNAi in larvae

L. salmonis dopamine receptor genes (LsDopamine1-2) were functionally assessed by RNAi experiment. Nauplius I larvae were exposed to LsDopamine1, LsDopamine2 and control CPY185 (cod trypsin) dsRNA fragments by soaking method. The experiments were terminated 7 days after soaking from nauplius I and analysed as copepodids. In order to evaluate the phenotype in each group, the lice were counted and observed under microscope. LsDopmine1 expression was down regulated above 90 % (Figure 5.14) in copepodids, but no significant effect on morphology and survival rate were observed compared to control group lice (Figure 5.13b). Further, fish infection trial with dsRNA treated LsDopamine1 copepodids showed similar

development pattern between groups (Figure 5.17) and no transcript down regulation in comparatively chosen preadult I female or in pre-infection separated copepodids were observed (Figure 5.18B, C). Three trials of RNAi were performed for LsDopamine2. In the first RNAi experiment, 60 % of the copepodids were dead at day 7 and distinguished phenoptype were seen in surviving larvae compared to control group copepodids larvae (Figure 5.12). About 75 % down regulation was detected in surviving larvae for LsDopamine2 (Figure 5.15A). Nauplius I larvae were treated again with LsDopamine2 dsRNA fragment to confirm the mortality rate in a first trial and to measure down regulation in the early stage before they start to die as copepodids. Hence, nauplii II were harvested two days post incubation and qRT-PCR showed up-regulation in RNAi lice compared to control group (Figure 5.15B), whereas copepodids in a third repeat experiment did not show any significant phenotype or down regulation (Figure 5.13c and 5.16). Mortality rates for control CPY185, LsDopamine1 and LsDopamine2 (repeat experiment) group, were calculated as 1.8 % and 2.6 % and 2.4 % respectively.

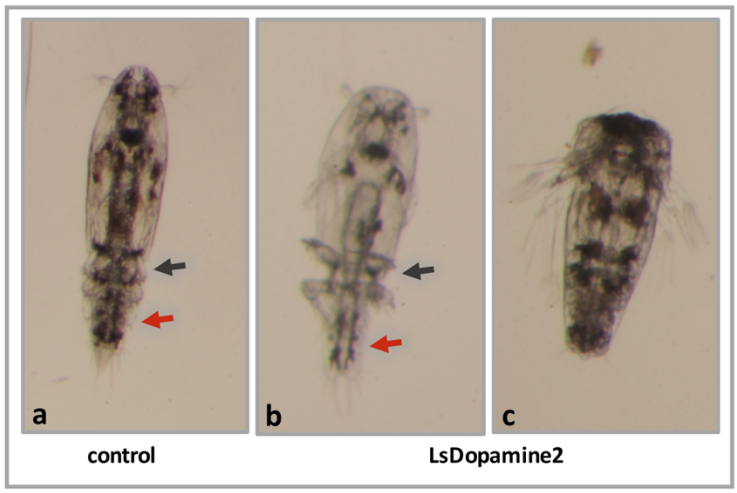

Figure 5.12: Morphological difference between control and knockdown LsDopamine2 copepodids. Black arrows indicate abnormal positioning of legs and red arrows represent abnormal tail appearance in the treatment group (b) compared to control group copepodids (a). Sample c was unable to molt into copepodids and looked abnormal compared to control group copepodids (a).

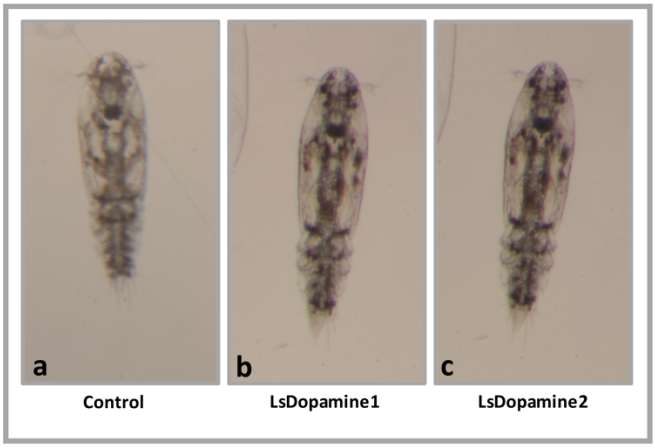

Figure 5.13: Copepodids with no distinguished phenotype. Common control group copepodid (a), LsDopamine1 group copepodid (b) and LsDopamine2 group copepodid (c) are shown with no morphological difference after RNAi experiment.

5.5.2 Quantification of transcriptionally regulated LsDopamine1-2 after RNAi

The expression of LsDopamine1-2 following RNA interference treatment was confirmed by qRT-PCR, 2-7 days post-incubation. Real time RT-PCR was performed on triplicates and five parallel sets of RNAi lice (LsDopamine1-2) and control (cod trypsin CPY 185) samples to determine the extent of regulation. Relative expression of LsDopamine1-2 was calculated using control lice samples of each group as calibrator. T-tests showed significant differences between control and RNAi lice samples for LsDopamine1 (Figure 5.14) and LsDopamine2 (Figure 5.15A). Over 90% reduction was observed in LsDopamine1 expression levels. In LsDopamine2 second and third repeat trials no significant difference was observed (Figure 5.15B and 5.16). In third repeat trial, the expression levels of LsDopamine2 were significantly higher in the replicates RNAi 2 and 4, and lower in RNAi 3 and 5 as compared to control samples (Figure 5.16A). Higher expression levels of LsDopamine2 in second repeat trial were also observed in RNAi sample compared to control sample (Fig 5.15B).

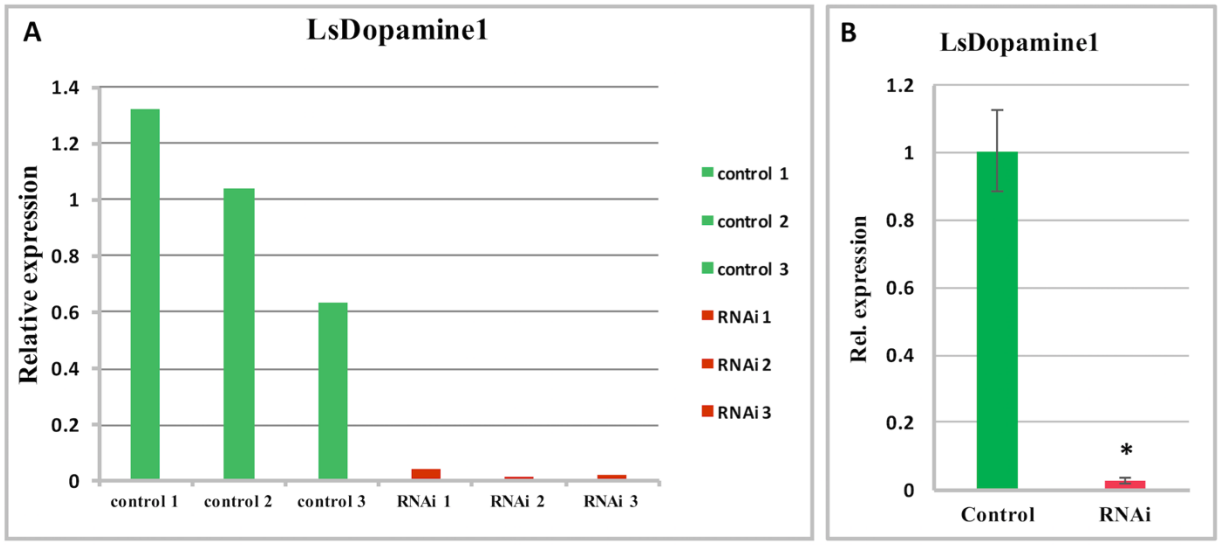

Figure 5.14: Relative expression of LsDopamineI in RNAi samples compared to control samples. Control group lice are represented by green bars whereas, lice exposed to dsRNA fragment are represented by red bars. Samples were taken after 7 days of incubation. (A) show expression levels for 3 parallels in copepodids. (B) Columns show mean expression levels and error bars represent confidence intervals as individual differences for each group. An asterisk (*) marks a significant difference between knockdown lice and control group (p= 0.008; t= 4.8).

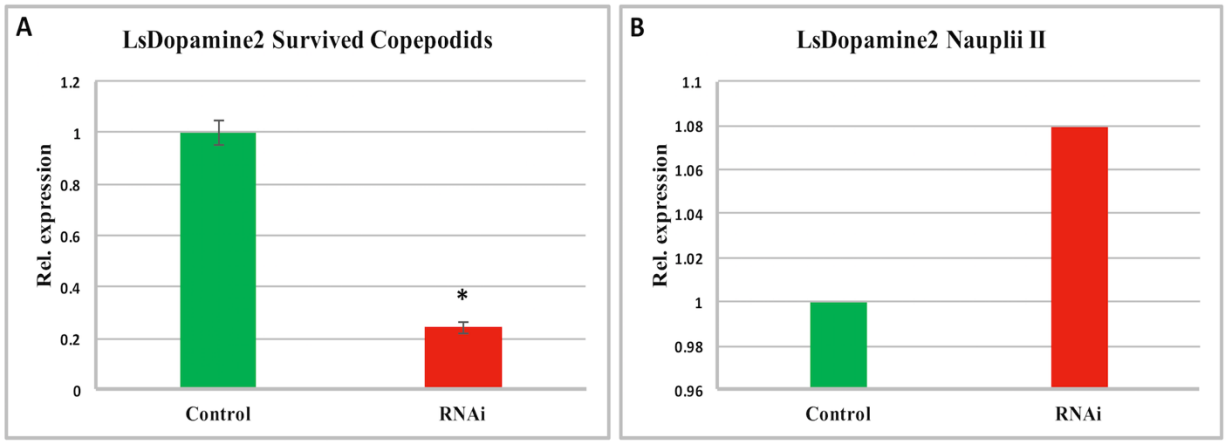

Figure 5.15: Varying transcriptional levels of LsDopamine2. Control group lice are represented by green bars whereas, lice exposed to dsRNA are represented by red bars. Samples were taken after 7 days of incubation (survived copepodids; A), 2 days of incubation (nauplii II; B). (A) columns show mean expression levels and error bars represent confidence intervals as individual differences for each group. An asterisk (*) marks a significant difference between knockdown lice and control group (p= 0.01; t= 7.5). Figure B show up regulation in nauplii II RNAi lice sample compared to control sample with a p value of 0.59.

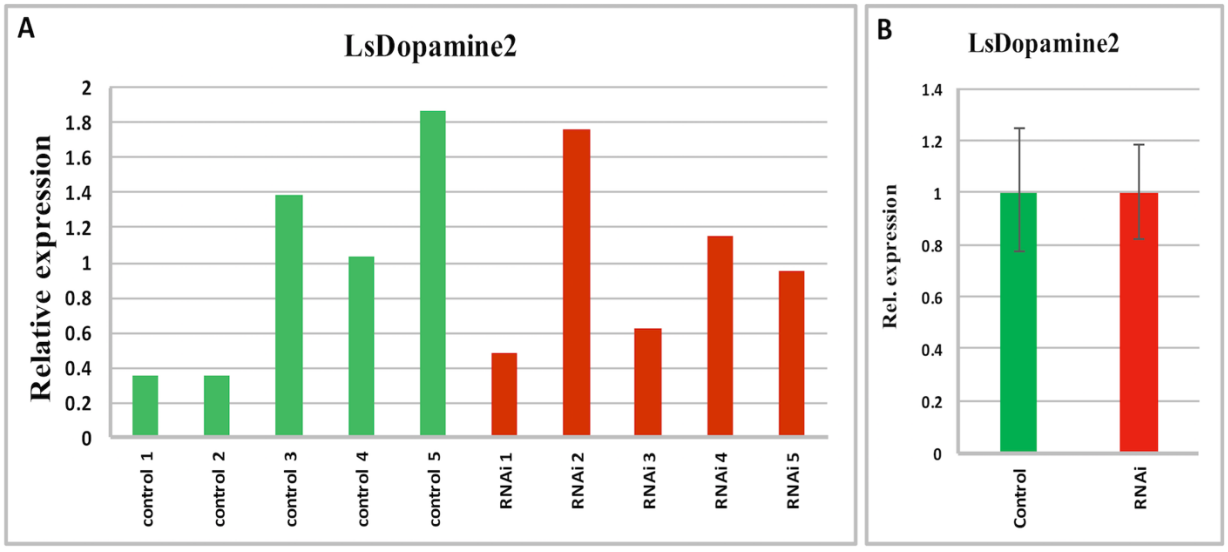

Figure 5.16: Relative expression of LsDopamine2 in RNAi samples compared to control samples. Control group lice are represented by green bars whereas, lice exposed to dsRNA are represented by red bars. Samples were taken after 7 days of incubation. (A) show expression levels for 5 parallels in copepodids (A). Columns show mean expression levels and error bars represent confidence intervals as individual differences for each group. A t-test shows no significant difference between RNAi and control samples (p= 0.1; t=0.01).

5.5.3 Experimental Analysis of Fish Infection with Copepodids

The fish infection experiment was conducted with RNAi treated copepodids for LsDopamine1 receptor gene and control (CPY 185) to check the effect on development pattern and phenotype of the lice.

Table 5.1: Data for Control and Experimental groups of lice in different phases of its life cycle

	Control I CPY 1	Control II CPY 1	LsDopamine D1 I	LsDopamine D1 II
Treated lice	100	100	100	100
Recovered lice	56	54	32	72
Chalimus II %	-	-	2 (6,25%)	-
Pad IM %	6 (10,6%)	8 (14,8 %)	4 (12,5%)	8 (11%)
Pad IF %	38 (68%)	23 (42,6 %)	18 (56,25%)	44 (61%)
Pad II M %	12 (21,4%)	23 (42,6%)	8 (25%)	20 (28%)

A total of 56 and 54 lice were recovered from the control group and high variation were observed in the number of lice recovered from the experimental (LsDopamine D1) groups i.e. 32 and 72 after the infection experiment. Number of treated lice, recovered lice and the percentage in the number of recovered lice stage i.e. Chalimus II, Preadult I male (Pad IM), Preadult I female (Pad IF) and Preadult II male (Pad IIM) from each group is shown in the table 5.1.

5.5.3.1 Length measurements

Development pattern of lice was observed 21 days post infection (21 DPI). The Total Length (TL) and Cephalothorax Length (CL) were determined for each group of lice in the pictures. Each group showed a similar development pattern except the LsDopamine D1 I group that contained two chalimus II lice. Overall no significant difference in phenotype was seen in each developed lice stage for control and LsDopamine1 group. Length measurements were calculated for Control and Experimental group lice by obtaining the minimum Cephalothorax Length (CL), maximum CL, average CL, minimum Total Length (TL), maximum TL, average TL and average CL/TL ratio for each stage of lice according to each group (Table 5.2-5.3).

Table 5.2: Length measurements for Control (I-II) group lice

	Min CL	Max CL	Avg. CL	Min TL	Max TL	Avg. TL	Avg. CL/TL
Control I							
Pad IM	1.84	2	1.94	3.35	3.86	3.61	0.54
Pad IF	2.17	2.43	2.27	3.6	4.2	3.9	0.58
Pad IIM	2.04	2.67	2.56	3.68	4.95	4.66	0.55
Control II							
Pad IM	1.87	2.05	1.97	3.11	3.96	3.57	0.55
Pad IF	2.12	2.47	2.29	3.55	4.32	3.94	0.65
Pad IIM	2.19	2.71	2.56	4.02	5	4.63	0.55

Table 5.3: Length measurements for Experimental (I-II) group lice

	Min CL	Max CL	Avg. CL	Min TL	Max TL	Avg. TL	Avg. CL/TL
LsDopamine1 I							
CH II	1.31	1.42	1.37	2.24	2.32	2.28	0.6
Pad IM	1.9	2.1	2	3.32	3.91	3.46	0.57
Pad IF	1.37	2.45	2.25	2.4	4.25	3.88	0.57
Pad IIM	2.5	2.7	2.6	4.54	4.86	4.69	0.55
LsDopamine1 II							
Pad IM	1.82	2	1.92	3.11	3.81	3.47	0.55
Pad IF	2	2.39	2.29	3.34	4.28	3.86	0.59
Pad IIM	2.43	2.66	2.53	4.08	4.61	4.25	0.59

The average CL/TL values indicate similar development pattern for preadult I male, preadult I female and preadult II male lice in each group. Comparatively similar preadult I female lice was chosen for qPCR analysis. Chalimus II (CH II), preadult I male (Pad I M), preadult I female (Pad I F), preadult II male (Pad II M) and comparable preadult I female (PadIF C) lice in each group with CL/TL length is shown in figure 5.17.

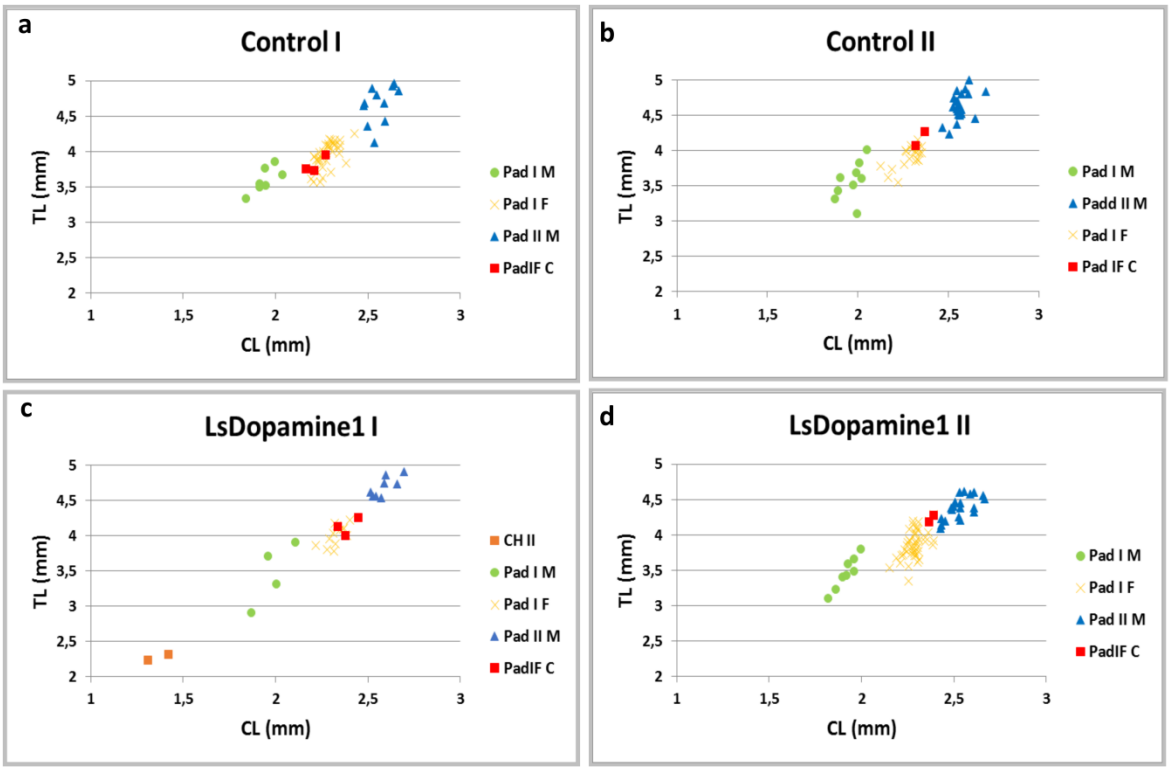

Figure 5.17: Length measurement and comparable pattern. Total Length (TL) and Cephalothorax Length (CL) of male and female lice were measured in imageJ software. In each group the preadult I male (Pad I M; green circles), preadult I female (Pad I F; yellow cross) and preadult II male (pad II M; blue triangles), in addition to chalimus II (CH II; orange squares) are represented with CL/TL values. Developmental pattern was observed in each group and preadult I female highlited in red squares were chosen for further qPCR analysis.

5.5.4 qRT-PCR Analysis of Pre-adult I Female Lice and Copepodids

In order to evaluate the effect of down regulated LsDopamine1 receptor gene in a fish infection experiment, a real time RT-PCR was carried out to measure the expression levels of LsDopamine1 in five biological replicates of comparatively similar pre-adult I female lice and pre-infection separated copepodids. The expression levels in knock–down (RNAi) lice samples were lower compared to control samples but the degree of down regulation was not significant (Figure 5.18B, C).

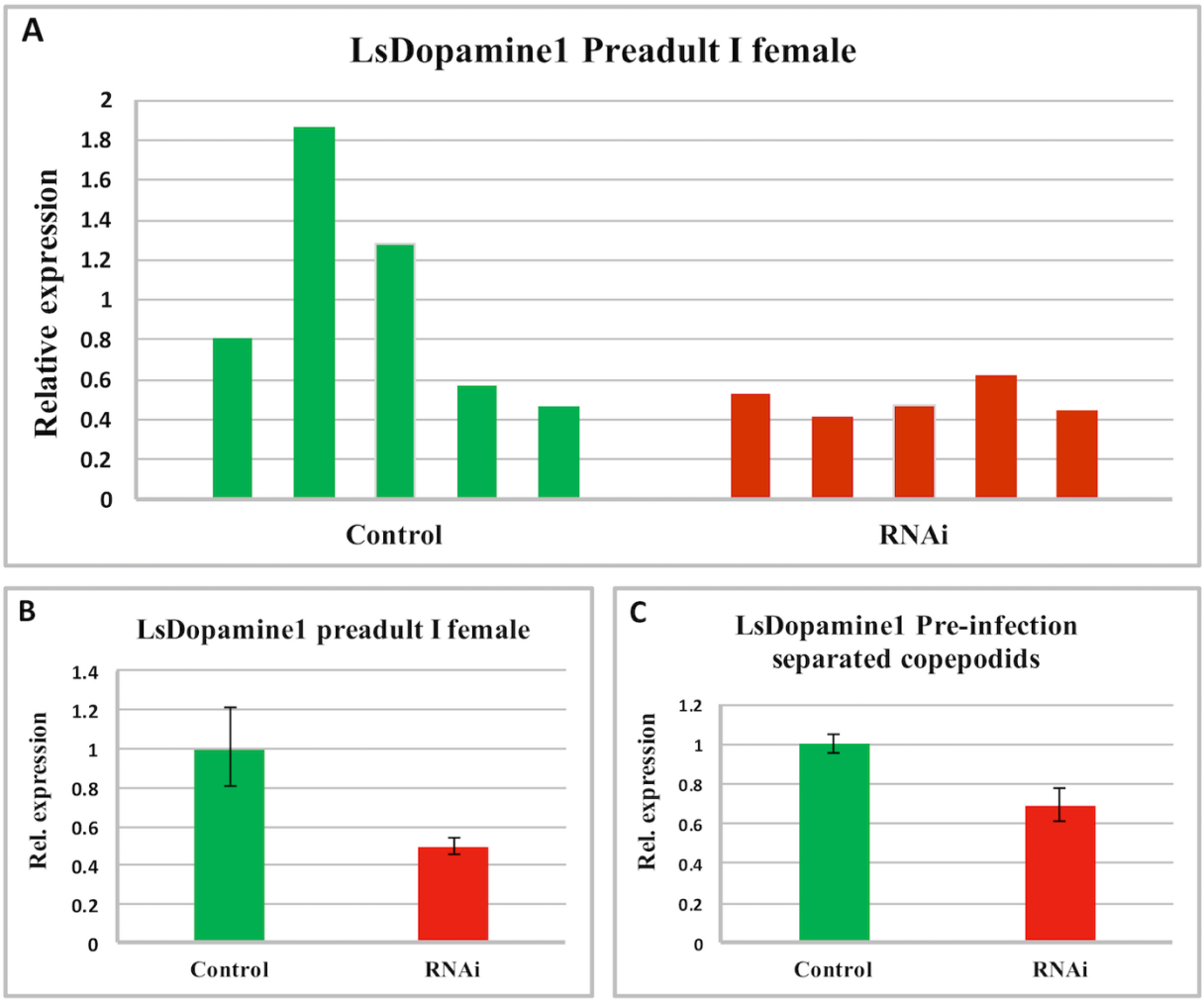

Figure 5.18: Representation of varying transcriptional levels of LsDopamine1 in preadult I female and copepodids. Control group lice are represented by green bars whereas, lice exposed to dsRNA fragment are represented by red bars. (A) Columns show expression levels in preadult I female for five parallels with single lice (samples were taken 21 days post infection). (B) Cumulative expression levels for preadult I female with control lice as a calibrator. (C) show mean expression levels for copepodids separated pre-infection experiment (samples were taken after 7 days of incubation). Error bars represent confidence intervals as individual differences for each group. A t-test show no significant difference ((B) p=0.09; t= 1.9, (C) p= 0.2; t= 1.8).

6. Discussion

In the present study, a total of eight *L. salmonis* genes were retrieved from LiceBase and two of them were identified as suitable candidates for dopamine receptor genes i.e. LsDopamine1 and LsDopamine2. Cloning of dopamine receptors was first performed by Bunzow et al. (1988) on mammalian D_2 dopamine receptor cDNA which opened a broad area in dopamine receptor research. Sequencing of the genes and cDNAs for LsDopamine1-2 have provided information regarding their structural features and their possible involvement in physiological functions. LsDopamine1 retrieved from LiceBase did not show any intron in the cDNA sequence and verified by PCR (Figure 5.1). RACE was performed to obtain a full length sequence of the transcript of LsDopamine2. LsDopamine2 consists of eight exons, seven introns and two splice variants derived by alternative splicing (Figure 5.2). These splice variants are a combination of the same exons which indicate a regulation by the same promoter. The splice variants are assumed to be differentially expressed as per the environmental and developmental requirements. In *Drosophila* and *C. elegans*, D2-like dopamine receptors have been characterized with splice variants (Gotzes et al., 1994, Hearn et al., 2002). Whereas, mammalian dopamine D2 receptors are reported to consist of 8 exons with short and long splice variants (Missale, 1998). The predicted complement of the transcripts of two *L. salmonis* dopamine receptor genes are differentiated as LsDopamine1 and LsDopamine2 mainly according to the presence of particular introns in the coding sequences and their respective large and short third intracellular loops and carboxy tails.

Dopamine receptor belongs to large family of G-protein coupled receptor (GPCR) and members of this family show considerable amino acid conservation in their seven transmembrane domains (Missale, 1998). Bioinformatics analysis of the domains and structure prediction (Figure X6-7) of LsDopamine1-2 showed that these receptors belonged to the similar seven-transmembrane domain family of rhodopsin like GPCRs. LsDopamine1 is predicted to consists of 354 amino acids and seven transmembrane (TM) domains while LsDopamine2 cDNA predicts a 450 amino acids protein and domain prediction analysis displayed five transmembrane domains. Apparently the first two TM domains were missing and N-terminal region was incomplete (Figure 5.3).

Technical difficulties pertaining to RNA purity for cDNA synthesis and time constraints could not render 5' RACE and PCR useful for confirming the start site for this particular transcript.

The dopamine receptors are further classified on the basis of their structural differences and functionally conserved residues in their transmembrane domains identified by the sequence alignment of LsDopamine1 (LsDOP1) and LsDopamine2 (LsDOP2) (Figure 5.4C). Initial BLAST analysis with candidate genes revealed highest similarity of LsDopamine1 and 2 with arthropods (*Drosophila melanogaster* and *Apis mellifera*). Further multiple alignment using CLUSTAL positioned LsDopamine1 closest to the sequence of *Aedes aegypti* (AeDOP1) and LsDopamine2 with that of *Drosophila melanogaster* (DmDOP2). TM domains are localized and functionally conserved residues such as aspartic acid (D) is found in TM domains II and III, two serine residues (SS) in TM domain IV and phenylalanine residue (F) in TM domain VI which are believed to be involved in dopamine binding. LsDopamine1 exhibit a short third intracellular loop (IL3) compared to a longer one in LsDopamine2 (Figure 5.4A, B). The intracellular loops are understood to be responsible for signaling and G-protein coupling (Macey et al., 2004). LsDopamine1-2 express two cysteine residues (C) in extracellular loops 2 and 3 (Ex 2, Ex 3) which is conserverd in all G protein coupled receptors (O'Dowd, 1993, Jackson and Westlind-Danielsson, 1994). These conserved cysteine residues are involved in forming disulphide linkages for the stabilization of receptor structure. Highly conserved residues close to TM domain V and TM domain IV are reported to interact with G-proteins (Wess, 1997). Conserved "DRY" motif found in the cytoplasmic region of TM III of both receptors (Figure 54A, B) known to be associated with G-protein coupling (Dixon et al., 1987, Fraser et al., 1988). Glycosylation sites are not found in LsDopamine2 amino-terminus as the first two TM domains are missing. Although LsDopamine2 have the presence of phosphorylation sites in the third cytoplasmic loop and on carboxy (-COOH) terminus. Serine residue on carboxy tail of LsDopamine2 are known to be phosphorylated by protein kinases (Missale, 1998). However, in rat D_{2L} dopamine receptor, similar protein kinases mediated phosphorylation of the serine residues have been shown to be involved in internalization and desensitization (Namkung and Sibley, 2004).

Phylogenetic analysis clusters LsDopamine1 along with insects i.e *Aedes aegypti* (AeDOP1) and *Apis mellifera* (AmDOP2), whereas, LsDopamine2 had the closest evolutionary relationship to arthropods such as *Panulirus interruptus* (PiDOP2) and *Drosphilla melanogaster* (DmDOP2) (Figure 5.5). Clustering of LsDopamine1 with AmDOP2 suggests that dopamine D1 and D2-like receptors have sequence similarities that could be structurally similar. However, some of the branch lengths have relatively low support value (i.e. below 50) which clearly indicate uncertainty in the branches.

qRT-PCR was performed to analyze the expression levels of LsDopamine1 and LsDopamine2 in nauplius I+II (young, middle, old), copepodids, chalimus I+II, preadult I+II, unmature adult female, adult male and adult female stages of the salmon louse (Hamre et al., 2013). To analyze the variation in gene expression before and after molting, LsDopamine1-2 expression levels were measured in young, middle and old instar ages of nauplius I and II. Expression levels of LsDopamine1 in nauplius I instar ages were seen in a decreasing pattern whereas, expression levels varied greatly in nauplius II instar ages. LsDopamine2 expression levels in nauplius I-II instar ages followed a similar pattern except nauplii II middle. qRT-PCR analysis points out to high expression of LsDopamine1 in adult male, followed by similar expression levels in planktonic and parasitic copepodids and nauplii II (middle). In the remaining stages of *L. salmonis*, LsDopamine1 expression was varyingly regulated (Figure 5.10). This pattern of expression levels in salmon lice life cycle follows a somewhat similar trend as the expression level retrieved from LiceBase for LsDopamine1 (appendix Figure X1). The highest level of LsDopamine2 expression was observed in planktonic copepodids followed by parasitic copepodids and nauplius I+II where smaller peaks of expression were analyzed in adult and preadult female with a little variation in rest of the stages (Figure 5.11). Expression pattern for LsDopamine2 in several *L. salmonis* developmental stages is also comparable with LiceBase expression levels with an increasing trend from nauplius to copepodids (appendix Figure X2). Highest expression levels of LsDopamine1 in adult male and LsDopamine2 in planktonic copepodids could be indicative of dopamine receptor roles in influencing the feeding and sexual behavior patterns of *L. salmonis*. Copepodids need food to grow to survive on host after their

survival on yolk nutrients during the earlier stages. The planktonic stage is a precursor stage to the parasitic stage, which could make a case for the up-regulation of the expression of LsDopamine2 for directing the organism to initiate feeding. The stimulus for adult males could be potential mating or a search for mating partner. It can be hypothesized based on the findings that a spike in expression of structurally different dopamine receptors (LsDopamine1 and 2) could have functional relevance in responding to a corresponding rise in dopamine levels which in turn could be a result of the organism's feedback response to environmental and developmental requirements. Further studies into the nature of dopamine and dopamine receptor expression can bring clarity into a detailed functional relevance of the same. It can also bring to light how factors like size and age interplay into metabolic rates which could therefore affect expression of certain genes. It also remains to be seen if technical variations as collection of samples at varying time points could affect the degree of regulation of these genes.

Dopamine acts as a neurotransmitter and dopamine receptors play an important role in the development, signaling and in control of behavior of insects (Nassel and Elekes, 1992). *In situ* hybridization was performed to localize the LsDopamine1-2 transcripts in copepodids and preadult I female louse. In copepodids, both LsDopamine1 and 2 transcripts are found to be widely distributed in the subcuticular tissues around the central nervous system of the *L. salmonis* (Figures 5.6 and 5.8). Subcuticular tissue is a tissue type presented by different cells and is organized in an irregular pattern throughout the lice along the cuticle. Up-regulation of the metabolic processes generally occur in the subcuticular tissues and the nutritional amino acids are transported directly to these tissues that are used by the cells of the body. In *C. elegans* and mammals, dopamine D2-like receptor has been shown to be expressed in several neurons including dopaminergic neurons and presynaptic nerve cells which mainly function as an autoreceptor, thus regulating the release of dopamine (Mercuri et al., 1997, L'Hirondel et al., 1998, Jayanthi et al., 1998, Nass et al., 2002). D2-like dopamine transcript in *Drosophila* is expressed during the development process (larva and pupa) and in the adult head (Hearn et al., 2002), whereas the LsDopamine1-2 transcripts were detected in tegumental glands type I in anterior part of preadult I female louse (Figures 5.7 and 5.9). In ticks, exocrine glands are believed to be important in host-parasite interaction and facilitate secretion of intermediates that

modulate immune response of the host during feeding (Sauer et al., 2000). Willadsen (2006) found that these glands can be probable targets for designing vaccines that aim at inhibiting these secretions. Moreover, D1-Like dopamine receptor has been found to be highly expressed in tick salivary glands (Simo et al., 2014). Tegumental glands have been reported to function differently in crustaceans based on their localizations at different sites (Bannister and Herring, 1989). Adjacent slides were stained with hematoxylin and erythrosine staining HE (S) for identification of cell types. Tegumental glands are globular in shape and have cuticular pores (Figures 5.7C and 5.9C). HE (S) staining results in faint staining of nuclei and cytoplasm and the possible reasons might be due to the intensity of stain used or the time exposed to hematoxylin and erythrosine.

RNAi is a well established method for resolving functional relevance of differentially expressing genes in different organisms. However, little is known about systemic RNAi pathways in non-traditional model organisms except plants and *C. elegans* (Miller et al., 2012). A similar approach was adopted for salmon lice by Eichner et al. (2014). In the present study, RNAi was used in planktonic nauplius I larvae to assess the function of LsDopamine1-2 and decreasing gene expression was observed in copepodids. Knock-down of LsDopamine1 was confirmed by qRT-PCR which showed significant down regulation of the transcript in copepodids (above 95%) (Figure 5.14). However, although down regulation was significant, it did not show any substantial changes in behavior or mortality in the experimental group (LsDopamine1) compared to control group lice (Figure 5.13). On average, cumulative mortality rate was established at 2.2% in control and also in experimental groups. RNAi was induced in nauplius I larvae for LsDopamine1, which were then used (as copepodids) to infect the fish in order to evaluate the effect on development pattern and phenotype of the lice and in regards to interaction with the host. 100 copepodids were isolated pre-infection to be further analysed by qRT-PCR. Each fish was infected with 100 RNAi treated copepodids. After 21 days post-infection, highly variant number of lice were recovered for the LsDopamine1 I and II groups, 32 and 72 respectively (Table 5.1). The variation in numbers of lice could be due to lice loss during molting, host behavior or ingestion or damage to lice during handling. Overall, no significant difference in the development of preadult I male, preadult I female and preadult II male was observed but two lice

in the Chalimus II stage had not molt according to the established life-cycle timeline. Some lice were reported to be still in the Chalimus II stage 20 days post-infection (pers. comm. C Eichner, 2016). The reason for this statistically insignificant but biologically significant effect could be that the LsDopamine1 is functionally non-essential until the pre-adult stages. The average CL/TL values indicates very similar development pattern in control and experimental groups (Table 5.2-5.3). Comparatively similar preadult I female were chosen from each group as LsDopamine1 was known from previous studies to be highly expressed after copepodids as is summarized in LiceBase. However, the degree of down regulation in the preadult I female and pre-infection isolated copepodids was not found to be significant in this study which was confirmed by qRT-PCR analysis (Figure 5.18). The T-test did not show any significant difference with a p-value of 0.09 and 0.2.

On the other hand, in the first RNAi knock-dowm experiment of LsDopamine2 in nauplius I larvae about 75 % down regulation was observed in the surviving copepodids (Figure 5.15A). Down regulation of LsDopamine2 apparently induced a distinct phenotype such as abnormal positioning of copepodid legs and tail appearance. Moreover, in some of the nauplius II larvae the knock-down resulted in complete molting arrest at day 7 (Figure 5.12c). The mortality was found to be 60 % in experimental group as compared to control group copepodids. The experiment was repeated to confirm the mortality rate of the first RNAi trial with LsDopamine2 and to measure down-regulation in the nauplius stage II before they start to die. Nauplii II were collected 2 days post incubation and transcript levels were analyzed by qRT-PCR which showed up-regulation in nauplii II RNAi lice compared to control lice (Figure 5.15B). Down-regulation degree similar to the first experiment was not observed is also a fact to be considered which could be a result of change in the concentration of dsRNA that was used in RNAi repeat experiments. It was shown that changes in concentrations worked well in other genes (Pers. comm. C Eichner, 2016). Copepodids at day 7 did not show any significant phenotype and cumulative mortality was 2.1% in control and experimental group. The knock-down experiment was repeated a third time with five replicates and down regulation was measured by qRT-PCR. Down regulation was varying and again not as high as the first experiment in experimental groups as compared to the first experiment (Figure 5.16). Furthermore, in all the RNAi repeat

experiments, mortality rate was significantly lower than the first RNAi trial with LsDopamine2 but it should also be noted that down regulation of the LsDopamine2 was not high as the first trial. Campbell et al. (2009) reported RNAi in nauplius had distinct phenotypic changes and the animals were dead after 3 days. The above mentioned study employed a similar approach by causing interference in Prostaglandin E Synthase genes in nauplii II stage. The approach therefore could give credence to the assumption that the observed mortality rate and phenotypic changes could be a direct result of high knockdown of LsDopamine2 genes in nauplii I, which was not the case in the above mentioned repeat experiments. This of course can be validated only with repeating experiments considering careful control of knockdown parameters and further observation. If the above assumption cannot be confirmed in future experiments, then the obvious explanation for the high mortality could be factors like presence of air bubbles or phenotypic changes could be just an exception of deformed or mutant animals in a wider population of wild type organisms.

7. Conclusion and Future perspectives

Knockdown of LsDopamine1 was significant with no adverse effects on phenotype. Infection studies with knocked down LsDopamine1 indicate overall similar development pattern in *L. salmonis* except for two chalimus II lice which were not developed further. This is not unusual as it has been observed in previous experiments but also leaves a slight room for assuming the role of LsDopamine1 to be non-essential until the preadult stages as LsDopamine1 had the highest expression in adult male stage. Further studies, which can significantly knockdown LsDopamine1 in stages closer to adult may shed light regarding its function in *L. salmonis*.

For LsDopamine2, a significant probable knockdown with relative phenotypic change was observed in survived copepodids. A confirmation about the reliability of knockdown and hence the phenotypic changes can be made only by carrying out RNAi in nauplii II. The two repeat RNAi experiments did not achieve similar significant knockdown and hence it can be assumed that morphological changes as seen in first RNAi experiment, in case of a direct result, with LsDopamine2 was not seen. Repeat of experiments with a benchmark of 90% knockdown set with similar experimental conditions used for first RNAi experiment in other stages will be helpful in ruling out mortality due to other factors like air bubbles and establishing a functional role for LsDopamine2.

An understanding about how reward pathways unravel in *L. salmonis* as they progress can be studied in detail by extending the case-control studies to include more parameters and having parallels of same populations with treated and untreated, starved and fed groups for LsDopamine2 in copepodids. The case-control studies can be extended for LsDopamine1 in adults to understand if it has a significant role in the sexual behavior pattern by understanding the likelihood of its mating in case of a knockdown of LsDopamine1 in adult stages.

LsDopamine2 can be further characterized in terms of its splice variants as they could be expressed differentially according to their developmental and environmental requirements. Challenging the organism with conditions that are not favorable to their survival can reveal the expression of different splice variants.

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APPENDIX

Dopamine receptor Sequences

LsDopamine1

ATGAATGAATCAAACAGAATGTTAAATATATCAAATCTCAGCTGTGTCTATTGTTTAAATGACAATAACG
TTCCCACATTTAAACGGGGCTATTTATTTGCTATTCTAATTAGTTTTGGAGTAGCTACAGTTTTAGGAAA
CTCTCTCATCATAGTATCAGTTTTAAAAAAGAGATCACTTCATACAGCAACGAATTACTTCATAACATCC
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LsDopamine2

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Protein Sequence

LsDopamine1

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KKRSLHTATNYFITSLAIADFLVGLVVMPFSATYEAMDQRWIFGPDWCDIWHSMD
VLASTASILNLCVISLDRYWAITDPMTYPYKMTGKRAAILIVLVWVCSSVISFPA
IAWWRLVAENVPPEWKCPFTEDMGYLIFSSTVSFYGPLTIMVFAYAKIYKAALEH
TRSLKLGAKVLAATGGPDSTLRIHRGGGGRDNSQKSNNNDVHCTLVNRDKNSISF
SKKIMKFSKEKKAAKTLGTVMGVFIICWLPFFITNIISGLCSDCIANPDFIIQVV
TWLXWXNSGMNPAIYAYCSKDFRR

LsDopamine2

MPFSVYVLVNWGMWGLPKIACDFYIALDVICSTSSIFNLVAISIDRYYAVTAPIK
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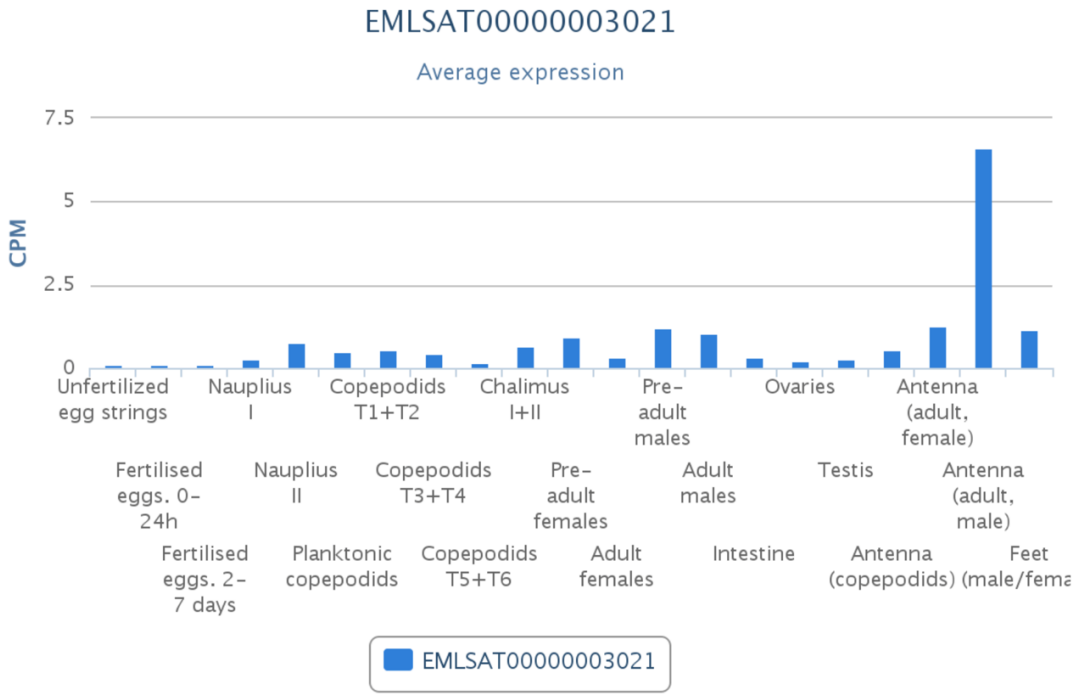

Figure X1: Expression levels of LsDopamine1 (EMLSAT00000003021) in L. salmonis developmental stages retrieved from LiceBase.

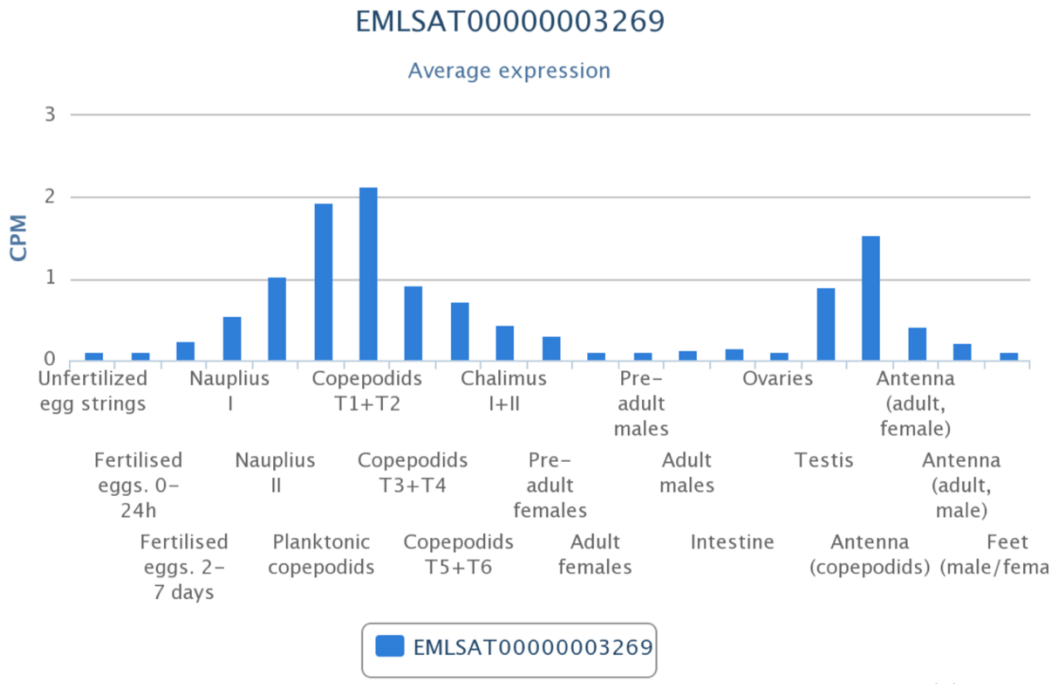

Figure X2: Expression levels of LsDopamine2 (EMLSAT00000003269) in L. salmonis developmental stages retrieved from LiceBase.

NCBI BLAST search

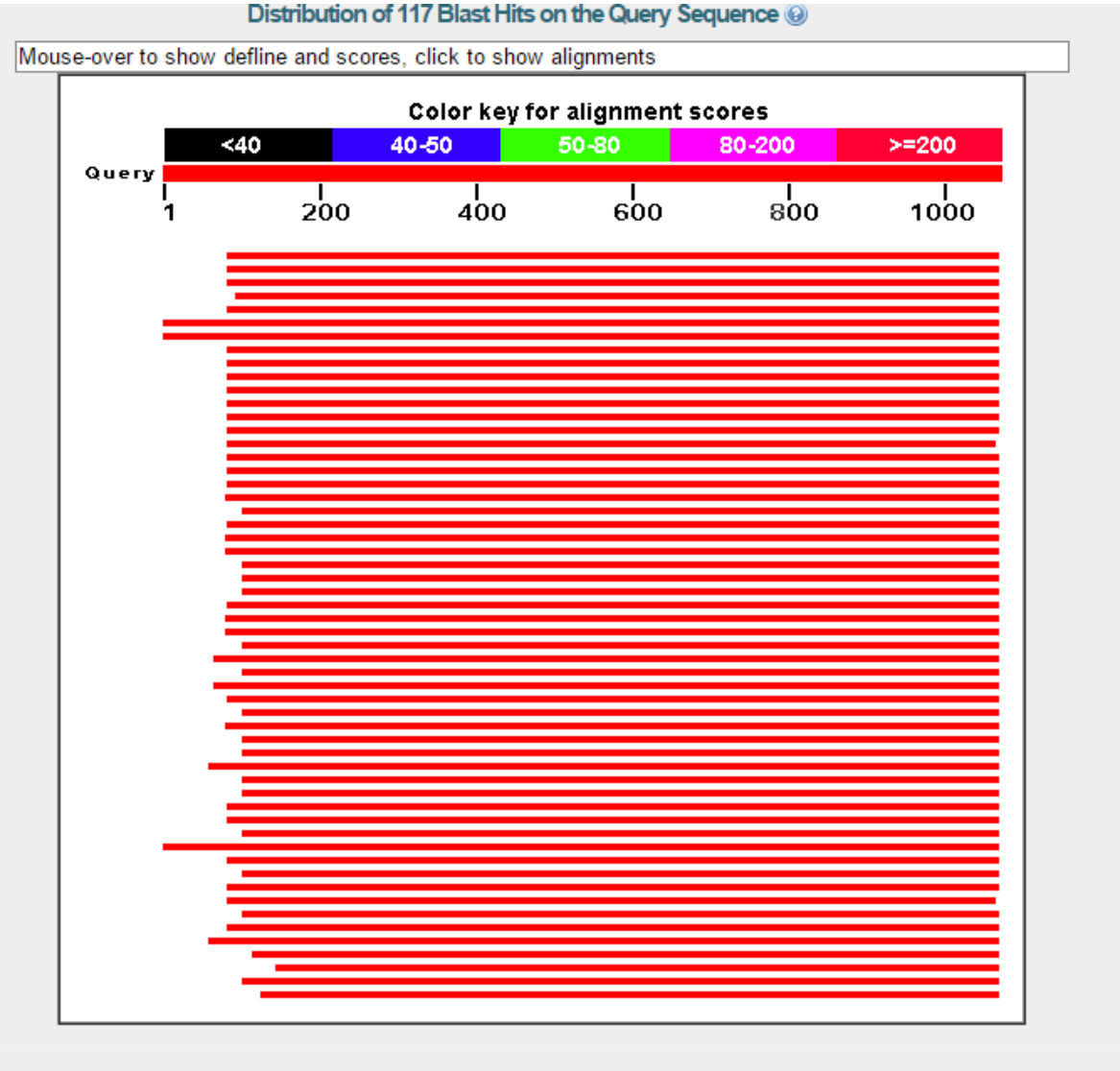

Figure X3: NCBI BLAST search of LsDopamine1 (EMLSAG00000003021).

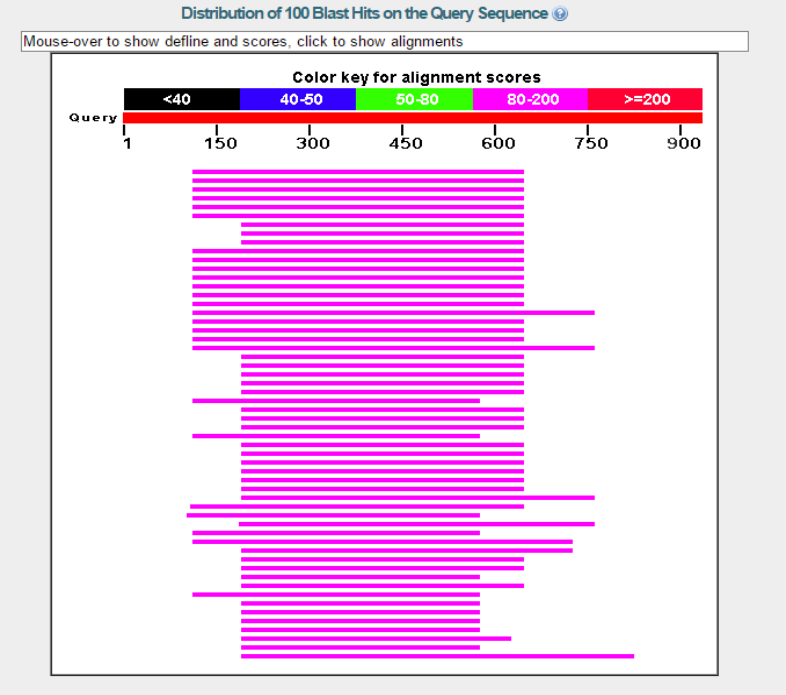

Figure X4: NCBI BLAST search of EMLSAG00000003269 displayed a partial receptor sequence.

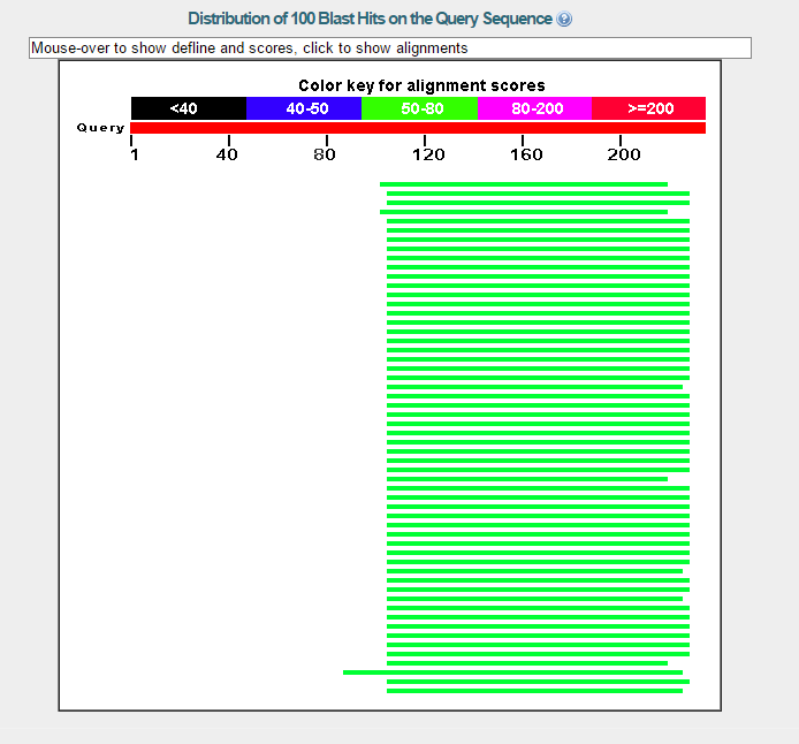

Figure X5: NCBI BLAST search of EMLSAT00000003268 displayed a partial receptor sequence.

Structure prediction

Figure X6: Structure prediction of LsDopamine1 was performed with I-TASSER

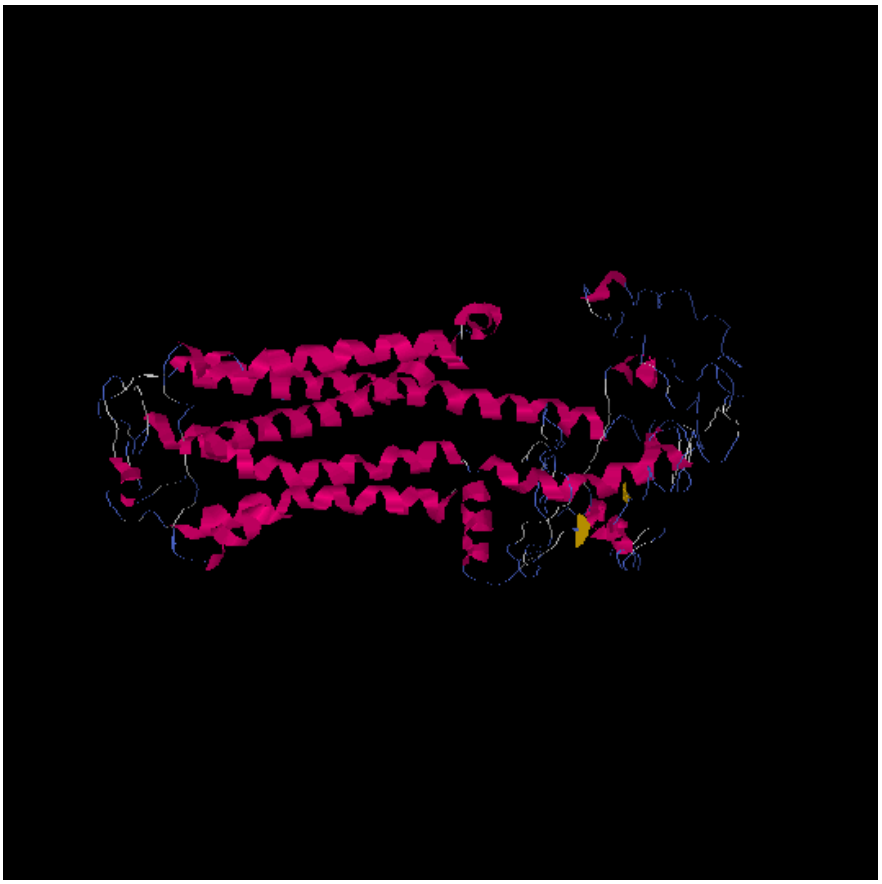

Figure X7: Structure prediction of LsDopamine2 was performed with I-TASSER