The effect of embryonic incubation temperature on the immune response of larval and adult zebrafish (*Danio rerio*)

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Preface

This thesis is submitted in fulfillment of the requirements for the degree of Philosophiae Doctor (PhD) at the Faculty of Biosciences and Aquaculture (FBA), Nord University, Bodø, Norway. The studies included in this thesis represent original research that was carried out over a period of four years from 10.08.2014 to 06.12.2018. The research was funded by the Norwegian Research Council (Ref. 213825), with additional support from Nord University (Norway).

The project team consisted of the following members:

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List of abbreviations

3’-UTR	3’ untranslated region
Ago	Argonaute
DE(G)	Differentially expressed (gene)
GO	Gene Ontology
HDL	High-density lipoprotein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon-regulatory factor
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
miRNA	microRNA
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nt	nucleotide
PAMP	Pathogen-associated molecular pattern
pri-miRNA	Primary miRNA
PRR	Pattern recognition receptor
miRISC	miRNA-induced silencing complex
scRNA-seq	Single-cell RNA sequencing
TCR	T-cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor

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List of papers

Paper I **Zhang Q**, Kopp M, Babiak I, Fernandes JMO. Low incubation temperature during early development negatively affects survival and related innate immune processes in zebrafish larvae exposed to lipopolysaccharide. *Scientific Reports* 2018, 8:4142.

Paper II **Zhang Q**, Babiak I, Fernandes JMO. Embryonic incubation temperature has a long-term effect on the immune transcriptome and its response to lipopolysaccharide in the spleen of adult zebrafish *Danio rerio*. Manuscript.

Paper III **Zhang Q**, Babiak I, Fernandes JMO. Thermal experience during early development modulates microRNA transcriptome in the spleen of adult zebrafish. Manuscript.

Abstract

In ectothermic animals, such as fish, temperature can have a profound effect in all physiological processes, including the immune response. In fish, multiple phenotypes show thermal developmental plasticity, which enables them to adapt to changing temperatures. However, little is known about the thermal developmental plasticity of the fish immune system and its impact on the immune performance of adults. We incubated zebrafish (*Danio rerio*) embryos at either low (24 °C), high (32 °C) or reference temperature (28 °C), and challenged first-feed larvae with lipopolysaccharide (LPS) at three challenge temperatures (24, 28, 32 °C) in a full factorial design. A low incubation temperature resulted in higher mortality rate compared to high or reference temperatures (**Paper I**). In addition, the mortality rate was positively associated with increasing LPS-challenge temperature. Transcriptomic analysis showed that similar immune transcripts were regulated by LPS at the low challenge temperature in fish incubated at low or high temperatures, but the enrichment of inflammatory processes was much higher in the high incubation temperature group (**Paper I**).

Both low- and high embryonic incubation temperatures had a long-term effect on the spleen transcriptome in adult zebrafish (**Papers II and III**). The expression of many immune transcripts, including cytokines, neutrophil- and T cell activity-related genes has been suppressed. In addition, a high diversity of immunoglobulin and complement component transcripts was induced in fish from the high incubation temperature group. Fish originating from the three incubation temperature groups showed distinct immune transcriptomes in response to LPS challenge. A large number of immune transcripts and processes was stimulated in fish from the low temperature group, whereas fish from the high temperature group showed a limited immune response at the transcriptional level and fish from the reference temperature group seemed to rely mainly on diverse apolipoprotein transcripts (**Paper II**). MicroRNA (miRNA) transcriptome analysis identified 33 differentially expressed (DE) miRNAs, including 30 (27 up-/3 down-regulated) DE

miRNAs in the spleen of fish incubated at high temperature compared to those kept at the constant reference temperature, and 3 (2 up-/1 down-regulated) DE miRNAs in fish kept at reference temperature after the LPS treatment. Enrichment analysis of potential target genes of DE miRNAs revealed similar immune processes and pathways to those obtained by direct analysis of the mRNA transcriptome (**Papers II and III**). Nonetheless, no DE miRNAs were identified in the spleen of fish incubated at low temperature compared to reference temperature group or in fish from low or high temperature after challenged with LPS. One possibility is that some miRNAs could be already stimulated by high embryonic incubation temperature before LPS challenge. Taken together, our data demonstrated that developmental temperature affects the immune plasticity of larval zebrafish but also has a long-term impact on the splenic immune transcriptome of adult fish. This is meaningful to understand the temperature-induced immune developmental plasticity in fish and is particularly relevant in the context of climate change.

1 Thermal regulation and plasticity

1.1 Thermal regulation in animals

Endothermic animals include mammals and birds, which have a constant body temperature maintained by internal metabolism regardless of the external thermal environment. Endotherms perceive external temperatures through cutaneous thermosensory proteins, mainly a large family of transient receptor potential ion channels, which detect and transmit afferent temperature signals to the hypothalamus (Bicego et al. 2007). The body core temperature is measured by the preoptic area and anterior hypothalamus, brain stem, and spinal cord, among which the preoptic area has an important role in integrating the temperature information from the local brain and that from other parts of the body (Boulant 2000). The sympathetic nervous system is then elicited, controlling a variety of autonomic thermoregulatory responses, such as cardiovascular response, cutaneous blood flow, skeletal muscle shivering, brown adipose tissue metabolism, cellular metabolic rate, sweating and panting. These physiological responses result in thermogenesis or heat loss, so as to maintain the body temperature at a constant level (Bicego et al. 2007). Besides, behavioral thermoregulation is an efficient method of adjusting the body temperature and is widespread among all the vertebrates studied so far. Behaviors such as basking in the sunshine and changing the body posture are quite common for many endotherms, while some other behaviors such as hibernation and spreading saliva on the fur are restricted to few species (Terrien 2011).

Ectothermic vertebrates include reptiles, amphibians, and most fishes. They cannot regulate the body temperature internally by metabolism adjustments thereby their body temperature fluctuates with the surrounding thermal environment. For thermoregulation, ectotherms primarily use behavioral strategies to thermoregulate, such as hibernation, basking and huddling/spreading the body. Some ectotherms have species-specific behaviors. For instance, the brooding Burmese python (*Python bivittatus*) coils around its eggs and uses spasmodic muscle contraction to elevate

body temperature for egg incubation (Brashears and DeNardo 2013). Whale sharks frequently ascend to the water surface after diving in the deeper and colder water for some time, in order to warm their body to the level required for physiological processes (Thums et al. 2013). Ectotherms have similar thermosensory pathways as endotherms, including cutaneous thermosensation, signal transmission to hypothalamus, and efferent sympathetic signals to regulate autonomic responses, such as cardiovascular response, and cutaneous blood flow (Seebacher 2009). Cardiovascular response, such as changes in blood pressure and heart rate is primarily mediated by central autonomic mechanisms but is also stimulated by nitric oxide and prostaglandins (Seebacher and Franklin 2004). The cardiovascular response exchanges heat by controlling blood flow between internal body and the surface, while skin vasoconstriction and vasodilation control cutaneous blood flow by heat convection between the body and environment (Dzialowski and O'Connor 2001; Seebacher and Franklin 2004).

Most teleost fish are ectotherms and they thermoregulate mainly through swimming to their preferential thermal areas. For instance, sockeye salmon (*Oncorhynchus nerka*) move to tributary plumes or the lake metalimnion when the temperature of the lake surface increases to physiologically suboptimal levels (Armstrong et al. 2016). In addition to warm/cool seeking, fish also show other thermoregulatory behaviors. For example, common carp (*Cyprinus carpio*) benefit from sun basking for warming their body above the ambient water temperature (Nordahl et al. 2018).

Some teleosts such as tunas (*Auxis rochei*, *Euthynnus affinis*, *Katsuwonus pelamis*, *Thunnus orientalis*, and *Thunnus thynnus*) and billfishes (*Xiphiidae* and *Istiophoridae*) are regional endotherms that use delicately arranged counter-current vascular structures to retain heat locally around some specific organs or tissues such as swimming musculature, brain, or eyes (Dickson and Graham 2004). Benefiting from this, regional endothermic fishes are able to locomote across a wide range of water temperatures and pursue prey with a burst of speed.

In 2015, the opah (*Lampris guttatus*), a tropical fish, was found to have total endothermy, which is the only whole-body endothermic teleost identified so far (Wegner et al. 2015). Their whole-body endothermy is achieved through two ways. First, the opah has the highest percent of aerobic pectoral musculature to body mass compared to all other fish species, which is insulated by a thick layer of fatty connective tissue. Thus, the metabolic heat is not only generated continuously when the fish is swimming, but also it is preserved from being lost to the surrounding water. Second, the counter-current vascular retia is located in the gills rather than in the swimming muscle or other parts of body, allowing the heat to be exchanged to cold efferent arteries from the gills and flowing to the whole body before convecting to the water temperature. As the result, the opah's body temperature is approximately 4.8 ± 1.2 °C higher than the ambient water temperature (Wegner et al. 2015).

1.2 Phenotypic plasticity

Organisms with identical or similar genotypes are able to develop different phenotypes in response to different environmental conditions; this phenomenon is termed phenotypic plasticity. It occurs at many different levels including behavior, physiology, or morphology. Temperature is a critical environmental factor, and temperature-mediated phenotypic plasticity is widespread among the vertebrates (Seebacher and Grigaltchik 2015; Noble et al. 2018), invertebrates (Bhardwaj et al. 2018; Clemente et al. 2018), or plants (Ibañez et al. 2017). In teleost fish, a variety of phenotypes show thermal plasticity, such as thermal acclimation capacity (Scott and Johnston 2012), muscle growth (Campos et al. 2014b), swimming performance (Oufiero and Whitlow 2016), developmental rate (Sparks et al. 2017), or cardiac mitochondrial metabolism (Ekström et al. 2017). Organisms experiencing temperature variations tend to have a higher plasticity to confront oncoming thermal challenges than those living at comparatively stable temperatures. For

instance, zebrafish eggs incubated at high (32 °C) or low (22 °C) temperature during the embryogenesis had better aerobic exercise performance in cold temperature (16 °C) than fish incubated at an intermediate embryonic temperature (27 °C) (Scott and Johnston 2012). Besides, temperature has various effects on different phenotypic traits. For example, the tropical reef fish *Acanthochromis polyacanthus* reared at a temperature 3 °C higher than the normal level showed reduced resting oxygen consumption, which is beneficial for them to save daily energy expenditure. However, these fish had smaller body length and poorer condition compared to those reared at the normal temperature (Donelson et al. 2011). In Alaskan sockeye salmon, a strong positive relationship between the thermal variation and phenotypic variability in developmental rate was observed, but other phenotypes such as body length and mass were largely insensitive to the experimental temperature in this cold-water fish species (Sparks et al. 2017).

1.3 Long-term thermal effect on biological functions in fish

Phenotypic plasticity, including early developmental plasticity and adult phenotypic acclimation, occurs throughout the life of most organisms. The consequence of developmental plasticity is not necessarily adaptive to the future environment, but also could be neutral or maladaptive (Fig. 1A, B), depending on the trend of changing environment and the generation time of an organism (Davis and Wund 2016). In the case of adaptive phenotypic variation, the environmental cue is a reliable predictor of the future environment, and the developmental plasticity increases the fitness of phenotypic traits to better match with the forthcoming environment (Fig 1A; Beldade et al. 2011). For instance, mosquitofish (*Gambusia holbrooki*) hatched in early spring may encounter both cool and warm temperatures; thus, they have a more plastic metabolic repertoire to maintain their performance in the oncoming variable temperatures. In contrast, fish hatched in early summer probably only experience warm temperatures, and become less flexible to cope with the coming

cool autumn (Seebacher et al. 2014). Adult phenotypic acclimation is generally reversible, such as seasonal fluctuation of the immune system in three-spined stickleback (*Gasterosteus aculeatus*) (Brown et al. 2016), and can compensate to some extent the mismatch between developmental plasticity-induced maladaptive phenotypes and the adult environment (Fig 1C). Whereas, the developmental plasticity is often irreversible if not all, such as temperature-induced sex determination in European sea bass (*Dicentrarchus labrax* L.) (Díaz and Piferrer 2015), and it determines the capacity of the thermal acclimation in adult organisms (Beaman et al. 2016).

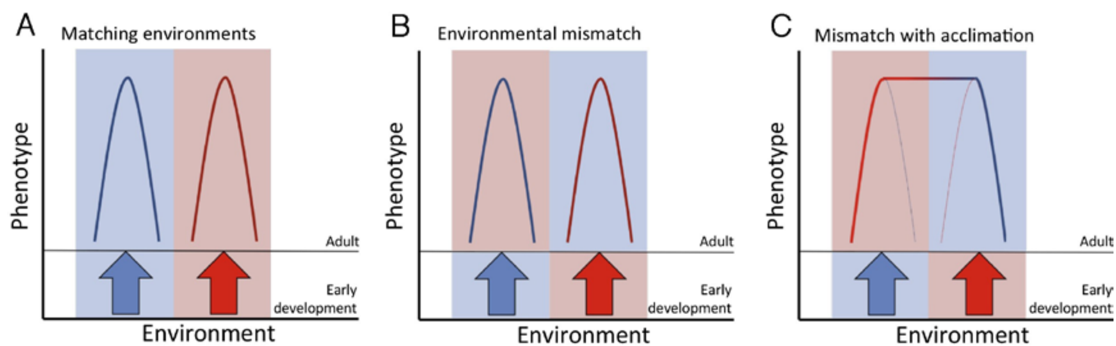

Figure 1. The relationship of early developmental plasticity and the environment experienced during the adulthood. Early developmental phenotypes may match (A) or mismatch (B, C) the adult environment, but reversible acclimation can reduce the developmental mismatch to some extent (C). (Beaman et al. 2016, RightsLink permission: 4476920710979).

In teleost fish, developmental temperature can have a persistent effect on a variety of phenotypes (Fernandes et al. 2006; Koumoundouros et al. 2009; Dimitriadi et al. 2018; Schnurr et al. 2014; Sfakianakis et al. 2011). However, little is known on the long-term effect of developmental temperature on the immune system of adult fish.

To the best of my knowledge, there is only a recent study on developmental plasticity of the fish immune system. It showed that the temperature during embryonic and larval development of gilthead sea bream (*Sparus aurata,* L.) had a long-term effect on the number of pronephric melanomacrophage centers and the level of *dopachrome tautomerase* (*dct*) transcript in adult fish (Mateus et al. 2017). Nonetheless, the expression of *dct* was examined under confinement stress rather than bacterial challenge, and other components of the immune system were not evaluated in that study.

2 The immune system of teleost fish

2.1 The innate immune system of teleost fish

The thymus, the kidney and the spleen are the three main immune organs in teleost fish, in addition to gut-associated lymphoid tissue, skin, gills, and the liver (Magnadóttir 2006) (Fig. 2A). The first immune line of defense in fish is the integumental barrier, composed of skin, gill, gut and the mucus covering them. It works as a physical barrier to prevent pathogens from invading the host and contains various antibacterial factors (Ellis 2001). In addition, teleost fish are able to initiate humoral and cellular immune responses to eliminate pathogens that breached the skin barrier. The humoral component includes antimicrobial peptides, lysozyme, lectins, complement, cytokines and natural antibodies, while the cellular response involves monocytes/macrophages, granulocytes/neutrophils, non-specific cytotoxic cells and dendritic cells (Magnadóttir 2006). Monocytes/macrophages and neutrophils are the main phagocytes that engulf pathogens, killing them directly by lysosomal digestion or/and respiratory burst (Silva and Correia-Neves 2012). Besides, other cell types such as dendritic cells, epithelial cells, endothelial cells, non-specific cytotoxic cells and lymphocytes also have phagocytic functions (Prame Kumar et al. 2018) (Fig. 2B).

The recognition of pathogens is achieved through their pathogen-associated molecular patterns (PAMPs) matched by the transmembrane pattern recognition receptors (PRRs) of immune cells. Toll-like receptors (TLRs) are the main class of PRRs capable of detecting various microbial components, such as flagellin, lipopolysaccharide (LPS) and lipopeptides (Kimbrell and Beutler 2001). To date, at least 20 TLRs have been identified in teleost fish, including 17 zebrafish TLRs (Meijer et al. 2004). Once stimulated, the TLRs pass down the signal through intracellular myeloid differentiation primary response 88 (MyD88)-dependent/-independent pathways, which further trigger the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB is a nuclear transcription factor that plays a central

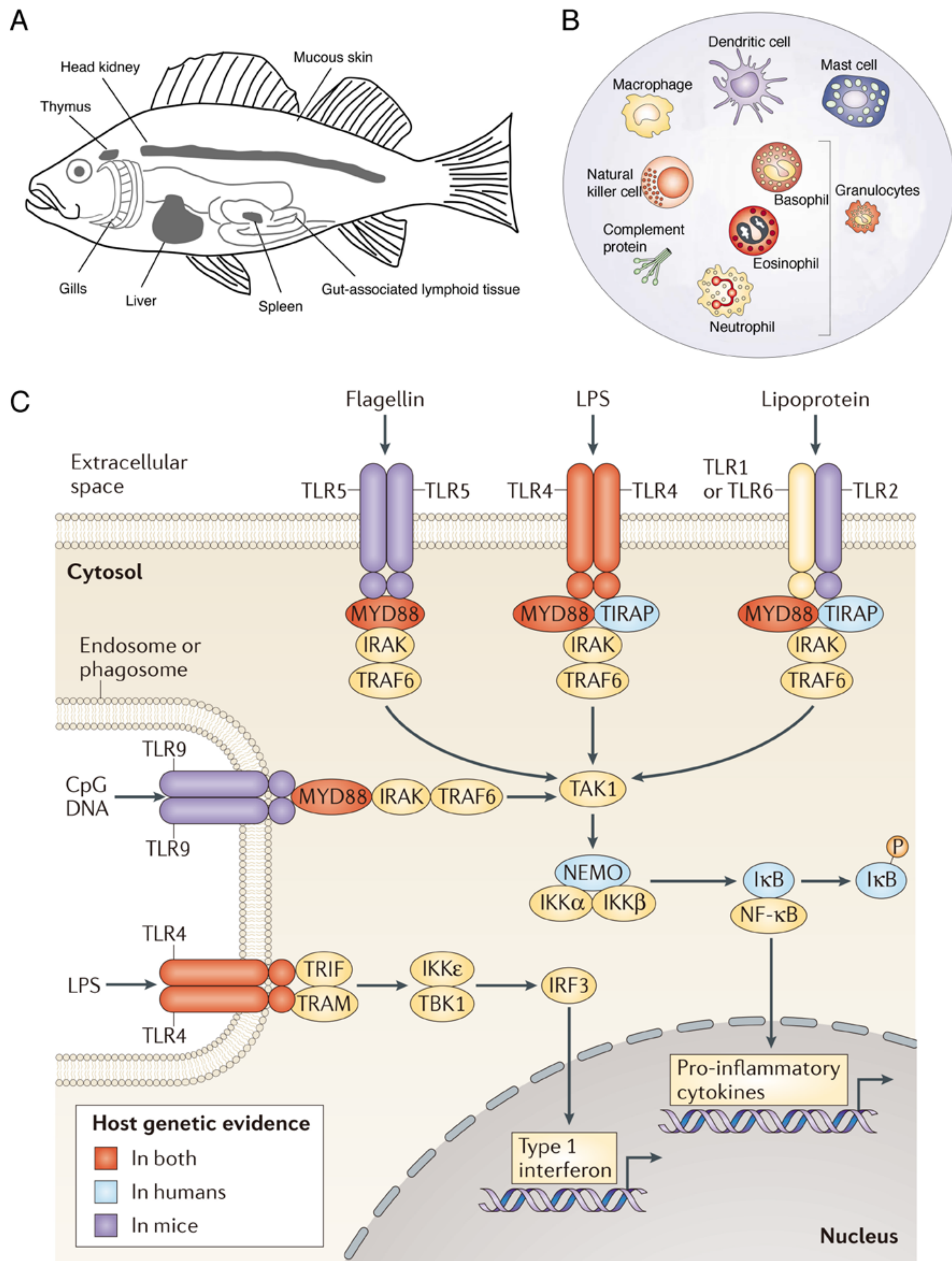

Figure 2. Teleost immune organs (A), mammalian innate immune cells (B, modified from (Dranoff 2004), RightsLink permission: 4480010077726), and TLR signaling pathway (C, (Gilchrist et al. 2015), RightsLink permission: 4480011436357).

role in regulating the inflammatory response. The activated NF-κB translocates to the nucleus, where it initiates the transcription of several key cytokine genes such as *tumor necrosis factor α* (*tnfα*), *interleukin 1 beta* (*il1β*), *il6*, and chemokine genes such as *il18*, *C-X-C motif chemokine ligand 1* (*cxcl1*), or *cxcl10* (Fig. 2C). These transcripts further stimulate more immune cells and attract them to inflammatory sites (Liu et al. 2017).

2.2 Adaptive immune system of teleost fish

The thymus provides the place for T lymphocyte differentiation and maturation, while pronephros (head kidney), corresponding to mammalian bone marrow, is the site for B lymphocyte development (Zapata et al. 2006; Sunyer 2013). Teleosts lack germinal centers and antibody class-switch recombination, only having three immunoglobulin (Ig) types (IgM, IgD, IgT/IgZ), while mammals have five (IgM, IgG, IgA, IgD, IgE) (Fillatreau et al. 2013; Sunyer 2013). Besides, there are no lymph nodes in teleost fish, but the spleen is an important secondary lymphoid organ that contains abundant mature B-/T-lymphocytes and myeloid cells (Trede et al. 2004; Zapata et al. 2006).

2.3 Environmental modulation of the immune system in teleost fish

2.3.1 Light

Different photoperiods are associated with Earth latitudes and revolutions. Disruption of a natural photoperiod may significantly affect physiological processes, including the immune response. For instance, immunosuppression with decreased polyclonal expression of T cells was observed in rainbow trout (*Oncorhynchus mykiss*) under the continuous light (Leonardi and Klempau 2003), while the shortened photoperiod (6L: 18D) resulted in recruitment of immune cells

(neutrophils and lymphocytes) in Piracanjubas (*Brycon orbygnianus*) as compared to the long photoperiod (18L: 6D) (Machado et al. 2016). Besides, reducing the light/dark cycle length may also have some effect on immune cells. Nile tilapia (*Oreochromis niloticus*) kept at a light/dark period of 6L: 6D had significantly higher amount of blood lymphocytes than fish kept at normal photoperiod (12L: 12D) (Biswas et al. 2004). In fact, photoperiod-sensitive circadian rhythmicity regulates immune parameters daily (Scheiermann et al. 2013). Another study showed that melatonin, a hormone secreted by the pineal gland under the tight control of clock genes, is involved in regulating the immune system in fish (Ángeles Esteban et al. 2006). For instance, complement activity was higher during the daytime than during the night in both gilthead seabream and European sea bass. In seabream, lysozyme activity peaked at night, while peroxidase activity was highest in the early morning (Ángeles Esteban et al. 2006). Some immune parameters such as peroxidase activity, phagocytic capacity, reactive oxygen intermediates (respiratory burst activity), or cell-mediated cytotoxic activity, as well as abundance of transcripts of IL1β, major histocompatibility complex (MHC), interferon-regulatory factor-1 (IRF1), IgM and T-cell receptors (TCRs) are enhanced by melatonin (Cuesta et al. 2008). On the contrary, melatonin can also suppress phagocytic activity in a dose-dependent manner, and reduce leukocyte chemotaxis, neutrophil numbers, CXCa chemokine expression and respiratory burst in inflammatory leukocytes (Roy et al. 2008; Kepka et al. 2015). This suggests that melatonin functions as an immune buffer (Carrillo-Vico et al. 2013), maintaining the homeostasis between pro- and anti-inflammatory responses.

2.3.2 Oxygen

Dissolved oxygen level is a limiting factor for fish immune defense. Hypoxia and reoxygenation can elicit oxidative stress (Zhang et al. 2016b), which is likely to impair antibacterial activity in fish (Boleza et al. 2001). In general, hypoxia reduces

the immune competence, such as the activity of respiratory burst (Ortuño et al. 2002) and lysozyme (Singh et al. 2016), the expression of TNFα, Interferon α (IFNα), IFNγ, Mx expression (Kvamme et al. 2013), and Ig levels (Scapigliati et al. 1999). Besides, severe hypoxia could cause fish death. For instance, sublethal hypoxia in channel catfish (*Ictalurus punctatus*) resulted in highly cumulative mortalities under the challenge with *Edwardsiella ictaluri* (Welker et al. 2007). Contrary to hypoxia, hyperoxia generally enhances fish immunity, even though it does not happen so often in the natural environment. For instance, Ig levels in European sea bass reared at hyperoxygenated sea water were two-fold higher than those in fish reared under normoxic conditions (Scapigliati et al. 1999). In farmed European sea bass, hyperoxygenation elevated the percentage of T-cells in gut, gills and thymus, and B-cells in peripheral blood leukocytes, head kidney and spleen (Romano et al. 2017).

2.3.3 Salinity

Salmonid fish, such as Atlantic salmon (*Salmo salar* L.), and euryhaline teleosts, such as gilthead seabream (*Sparus aurata*), translocate from freshwater to seawater or from brackish to an hyper-saline aquatic environment, and experience salinity concentration changes during this process (Talbot and Potts 1989; Bodinier et al. 2010). Salinity change also happens as a result of ocean current, inflow of rainfall, estuarine water, and ground water entering to the ocean.

The immune system of fish is significantly affected by the salinity change. For instance, after Atlantic salmon is transferred to sea water, a large number of immune genes in the head kidney, intestine, and gills are differentially expressed, most of which down-regulated, and involving functions of humoral and cellular innate immunity, inflammatory response, and antigen presentation (Johansson et al. 2016). However, another study in salmon showed that the skin barrier such as cutaneous secretion, mucus cell numbers, protein composition and immune activity were enhanced after the seawater transfer (Karlsen et al. 2018).

Acute salinity change is detrimental for fish, and salinity shock can cause fish death. For example, an acute transfer from fresh water to sea water caused significant enhancement of alternative complement pathway activity, phagocytic activity and respiratory burst activity in Mozambique tilapia (*Oreochromis mossambicus*) (Jiang et al. 2008), and marked increase of phagocytic activity of pronephric leucocytes and lysozyme concentrations in brown trout (*Salmo trutta*) (Marc et al. 1995). While an abrupt change of salinity from 33 parts per thousand to either 20 or 40 parts per thousand caused grouper fry (*Epinephelus* sp.) much more susceptible to viral infection and resulted in higher mortalities compared to fish only exposed to the virus (Chou et al. 1999). Relative to abrupt transfer, the gradual transfer to sea water induced lesser intensity and shorter duration of effects on the phagocytic activity and plasma lysozyme concentration in brown trout (Marc et al. 1995).

In general, neither low nor high but intermediate salinity is beneficial for marine fish immunity. For instance, turbot (*Scophthalmus maximus* L.) reared at moderate salinity of 20 parts per thousand had enhanced immunity, such as lysozyme activity, alternative complement pathway activity, phagocytosis, and highest survival rate after the bacterial challenge compared to counterparts from salinities of 8, 32 and 40 parts per thousand (Zhang et al. 2011). A similar result was observed in golden pompano (*Trachinotus ovatus*) (Ma et al. 2016). Another study in gilthead seabream demonstrated that low-salinity (6 parts per thousand) acclimation for 100 days or high-salinity (55 parts per thousand) acclimation for two weeks caused stress to fish, resulting in decreased peroxidase content and alternative complement activity, and increased plasma IgM level, respectively, compared to the control salinity (38 parts per thousand) acclimation (Cuesta et al. 2005).

2.3.4 Temperature

Temperature affects the immune response of fish extensively, and its consequences are quite variable depending on immune parameters, fish species and temperature

regimes (Morvan et al. 1998; Abram and Dixon 2017). For instance, sockeye salmon reared at 8 °C had a greater percentage of phagocytic kidney macrophages and higher complement activity compared to fish reared at 12 °C (Alcorn et al. 2002). Common carp acclimated at 12 °C possessed a higher level of respiratory burst, phagocytosis of pronephric macrophages and non-specific cytotoxic cell activity than fish acclimated at 28 °C (Le Morvan et al. 1996, 1997). High acclimation temperatures enhanced respiratory burst activity and lytic activity of complement pathways in rainbow trout compared to low temperatures (Nikoskelainen et al. 2004). Low temperature induced a less-extent activation of monocytes and granulocytes in bacteria-challenged rainbow trout compared to high temperature, even though similar immune activation was induced in both temperature conditions (Köllner and Kotterba 2002). The innate immune system can adapt to a new temperature and even to perform better at this temperature. Common carp acclimated at either low or high temperatures had higher cytotoxic activity of leukocytes in that acclimation temperature rather than in other temperatures (Kurata et al. 1995). This is consistent with the hypothesis of beneficial acclimation that organisms tend to perform better at the acclimation temperature than those without acclimation at this temperature (Wilson and Franklin 2002). The underlying mechanism could be that the thermal acclimation remodeled the physiological processes, including the immune system of animals, and shifted the temperature of maximal performance to the acclimation temperature (Seebacher et al. 2015).

The adaptive immunity tends to be suppressed by low temperatures and promoted by high temperatures. Juvenile European sea bass reared at 23 °C had higher IgM levels than their counterparts reared at 17 °C (Varsamos et al. 2006). Similarly, antibody production was lower in common carp acclimated at 12 °C compared to that of fish kept at 28 °C (Le Morvan et al. 1996). However, opposite results were also observed. After *Aeromonas salmonicida* infection, rainbow trout maintained at 10 - 12 °C had a higher amount of sera antibodies than those kept at 15 - 17 °C (Köllner and Kotterba 2002). The proliferation and cytotoxicity of lymphocytes were also positively associated with increasing temperatures. Sockeye salmon reared at

12 °C had a greater proportion of lymphocytes compared to fish reared at 8 °C (Alcorn et al. 2002). T lymphocyte proliferation decreased in common carp kept at low temperature compared to that in fish acclimated at high temperature (Le Morvan-Rocher et al. 1995). The specific cytotoxicity of lymphocytes was also lower in fish reared at low temperatures than their counterparts in fish reared at high temperatures (Fischer et al. 1999).

The immune system requires appropriate temperature regimes for optimal activity, and suboptimal temperatures may cause negative effects. For instance, the circulating IgM level in Nile tilapia was elevated following rearing temperature increase but declined when the temperature increased outside of the permissive temperatures for this species (Dominguez et al. 2004). In Atlantic cod (*Gadus morhua* L.) reared at 10 °C, the level of some specific immune transcripts in plasma such as β_2-microglobulin (β_2-M), MHC Class I, and IgM-L increased gradually from 10 to 16 °C, but returned to the original levels when the temperature continued to increase from 16 to 19 °C (Pérez-Casanova et al. 2008).

2.4 Immune transcriptome

2.4.1 mRNA

Understanding the pathological mechanisms in fish, particularly commercial fishes such as Nile tilapia, common carp or Atlantic salmon, is important for fish welfare and to avoid pathogen-caused economic losses in aquaculture (Valenzuela-Miranda et al. 2015; Zhu et al. 2015; Jiang et al. 2016). Transcriptome analysis is an effective and promising way of deciphering the genome-wide transcripts involved in disease and immune response in fish (Sudhagar et al. 2018) and to understand the divergent responses to pathogens between immune organs. For instance, in Atlantic salmon infected with infectious salmon anaemia virus, transcripts associated with non-specific innate immunity, inflammatory and antiviral responses were

overrepresented in the gills and transcripts with anti-viral and interferon function were abundant in the liver, while mRNAs with the role of regulating adaptive immune response and antigen presentation were particularly transcribed in the head kidney (Valenzuela-Miranda et al. 2015). Transcriptome analysis is promising in selecting more robust fish in response to pathogens. Through comparative transcriptome analysis between resistant and susceptible fish to pathogens with a genetically homogeneous background, a large number of differentially expressed genes have been observed in rainbow trout (Langevin et al. 2012), grass carp (*Ctenopharyngodon idella*) (Wan and Su 2015), and Atlantic salmon (Dettleff et al. 2017), laying the foundation for producing resistant fish. Moreover, the effect of some factors such as vaccination (Zhang et al. 2017b), nutrition (Martin and Król 2017), environmental adaptation (Huang et al. 2016), endothermy evolution (Marra et al. 2017) on the immune system have been investigated by transcriptomic analysis. In addition to microarray and the next-generation RNA-seq, single-cell RNA sequencing (scRNA-seq) is becoming a promising method for deciphering more accurate transcriptome of specific cell types (Carmona et al. 2017).

2.4.2 micro RNA

MicroRNAs (miRNAs) were first identified in *Caenorhabditis elegans* (Lee et al. 1993; Wightman et al. 1993) but they are now known extensively distributed among animals, plants, and microbes (Griffiths-Jones et al. 2008). In teleost fish, miRNAs are involved in regulating growth, organ development, reproduction and response to environmental factors (Bizuayehu and Babiak 2014). Most of mature miRNAs are generated through the canonical pathway (Fig. 3). Primary miRNA transcripts (pri-miRNAs) are originally transcribed from miRNA genes by RNA polymerase II or III. Then, pri-miRNAs are processed by Drosha, a nuclear RNase III, and DiGeorge Syndrome Critical Region 8 (DGCR8), a double-stranded-RNA-binding protein,

resulting in hairpin-structured precursor miRNAs (pre-miRNAs) of length ~70 nucleotide (nt). With the assistance of Exportin 5, pre-miRNAs are exported from the

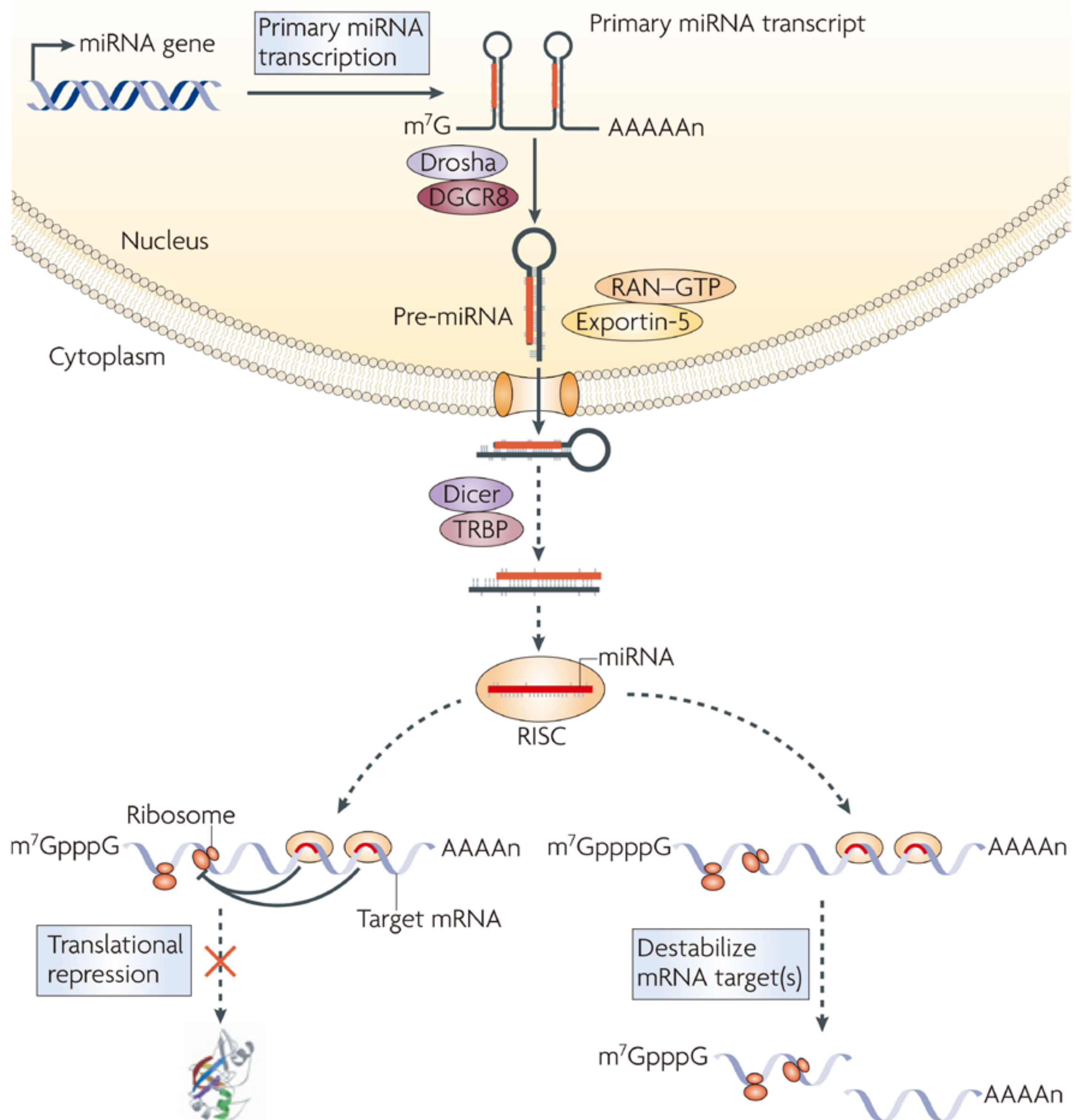

Figure 3. MicroRNA biogenesis and function in animal cells (Lodish et al. 2008). RightsLink permission: 4477040844183.

nucleus to cytoplasm, bound to Dicer-TRBP complex and cleaved into ~22 nt-length mature miRNA duplex. The miRNA duplex is subsequently loaded to Argonaute (Ago) proteins, assembled in precursor miRNA-induced silencing complex (pre-miRISC). Immediately after pre-miRISC formation, one strand of the miRNA duplex (passenger strand) is released, leaving the other strand (guide strand) together with Ago proteins in the mature RISC (Ha and Kim 2014). In addition to the canonical pathway abovementioned, non-canonical pathways can also generate miRNAs, in which the Drosha-mediated or Dicer-mediated processes are replaced by other mechanisms (Ha and Kim 2014).

Mature miRNAs regulate gene expression at the post-transcriptional level by guiding Ago proteins binding to the 3' untranslated region (3'-UTR) of target mRNAs. The domain from nucleotide position 2 to 8 at the 5' end of miRNAs is named "seed region" and determines Ago binding. The perfect Watson-Crick pairing between miRNA and mRNA leads to the degradation of mRNA, while the imperfect pairing results in the inhibition of translation (Ameres and Zamore 2013) (Fig. 3).

miRNAs are rather conserved among vertebrates especially within the seed sequence. One miRNA is able to regulate multiple mRNAs, and one mRNA can be targeted by many miRNAs (Ameres and Zamore 2013). Growing evidence suggest that miRNAs are involved in teleost immunoregulation (Andreassen and Høyheim 2017). For instance, TLR-mediated NF-κB pathway induces the expression of miRNAs such as miR-9, miR-146a, and miR-155, which in turn modulate the expression of pathway components such as MyD88, TNF receptor-associated factor 6 (TRAF6), and NF-κB1 (He et al. 2014; Zhou et al. 2018). miRNAs have dual functions in immunoregulation. On the one hand, miRNAs have increased expression in the response to bacterial stimulus, and positively regulate the expression of cytokines to elicit the inflammatory response (Wu et al. 2012). On the other hand, miRNAs function as negative regulators of several key components of TLR-mediated NF-κB

signaling pathways to avoid the excessive inflammation (Chu et al. 2017). Fish miRNAs can also be utilized by a virus for suppressing the host immune response and favoring viral invasion (Zhang et al. 2016a).

Thermal plasticity of miRNA expression has been reported in larval fish (Campos et al. 2014a; Hung et al. 2016; Zhang et al. 2017a), and this transcriptomic plasticity can be retained during the ontogeny (Johnston et al. 2009; Bizuayehu et al. 2015). However, it is still unclear whether the long-term effect of early developmental temperature on miRNA transcriptome is involved in regulating the immune response in fish.

3 Fish and challenge models used in the present study

3.1 Zebrafish as an immunology model

Zebrafish is a eurythermal teleost, with natural occurrence in Pakistan, India, Nepal and Bangladesh and is distributed in a wide temperature range (Engeszer et al. 2007) (Fig. 4A, B). The recorded living temperatures for zebrafish ranged between 16.5 and 38.6 °C in the wild, and 6.2 - 39.2 °C in the laboratory (López-Olmeda and Sánchez-Vázquez 2011) (Fig. 4C). Zebrafish has several advantages as a model organism, including being ectothermic, possessing both innate and adaptive immune systems, short generation time, low cost, easy maintenance, available mutants and a well-annotated genome, which make it an ideal candidate for evaluating the effect of external temperature on the fish immune system.

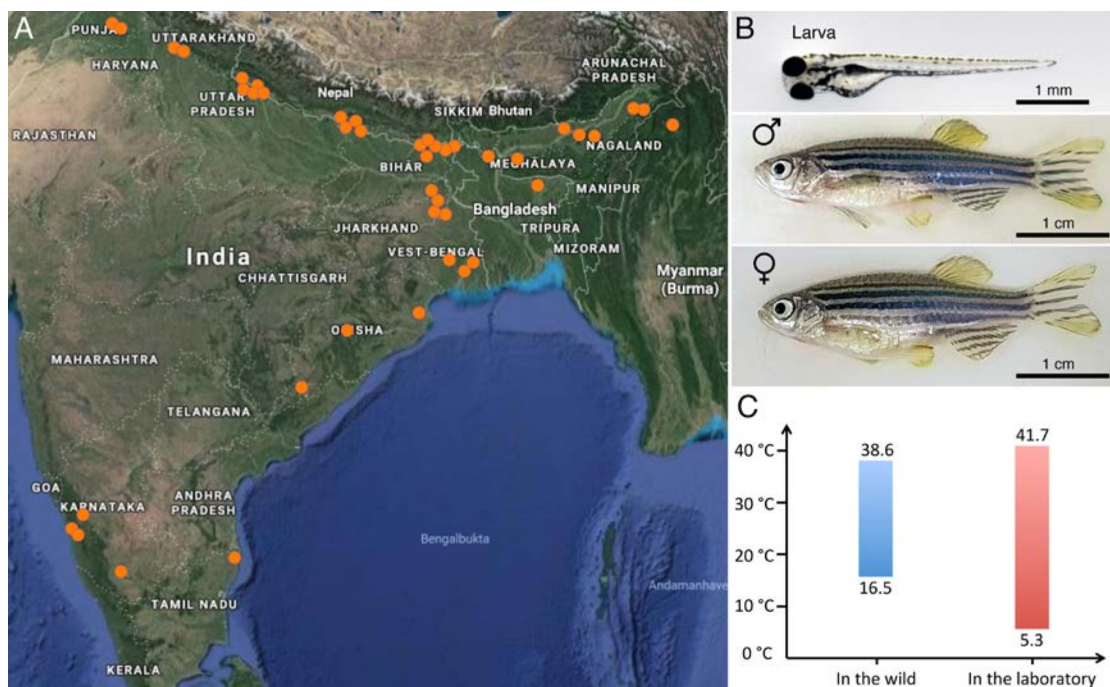

Figure 4. Zebrafish natural distribution (A), representative larva and adult stages (B), and the temperature ranges recorded in wild and laboratory populations (C).

3.2 Lipopolysaccharide as a Gram-negative bacteria mimic

LPS is the outer membrane component of Gram-(-) bacteria, capable of eliciting endotoxin shock in the host (Fig. 5A). In mammals, LPS is recognized by TLR4 with the aid of LPS-binding protein, myeloid differentiation protein 2 and CD14, which further stimulates MyD88-dependent NF-κB signaling pathway, initiating inflammatory gene expression. The LPS-TLR4 complex can also stimulate MyD88-independent pathway, enhancing the inflammatory response and activating type I interferon gene expression (Pålsson-McDermott and O'Neill 2004; Kagan 2017) (Fig. 2C). In addition to TLRs, there are a variety of different types of LPS receptors in mammals, including caspase, integrin, and G-protein-coupled receptor families, which make mammals highly sensitive to LPS (Kagan 2017) (Fig. 5B). In contrast, fish are quite tolerant to LPS due to a deficient extracellular structure of TLR4 (Sullivan et al. 2009). Some other alternative LPS-receptors, such as scavenger receptor and beta-2 integrins, have been suggested in fish (Iliev et al. 2005). Besides, high-density lipoproteins (HDLs) particularly apolipoproteins also have important roles in neutralizing LPS toxicity (Concha et al. 2004; Magnadóttir and Lange 2004). Despite the incomplete understanding of mechanisms underlying LPS recognition in fish, the LPS-induced inflammation signaling pathway is quite conserved compared to mammals (Forn-Cuní et al. 2017), and many studies have been carried out using LPS as bacterial mimics (Yang et al. 2014; Jiang et al. 2015; Liu et al. 2016). Therefore, in the present thesis we chose LPS as a challenge model of Gram-(-) bacteria.

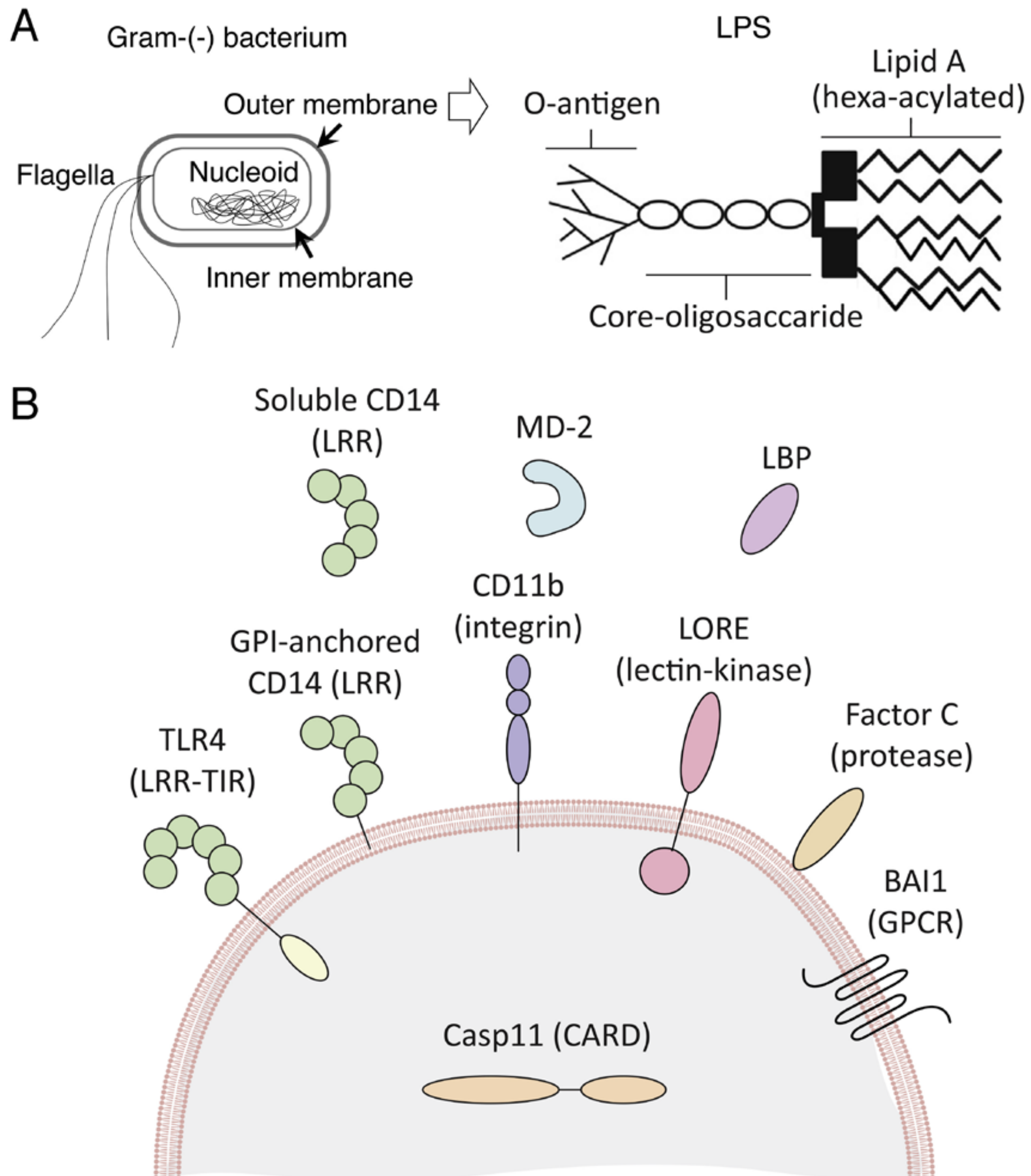

Figure 5. Lipopolysaccharide (LPS) structure (A), and diverse receptors in plants and animals (B). LPS receptors include TLRs, integrins, G-protein coupled receptors (GPCRs), proteases and kinases. Receptors can be membrane proteins, soluble cytosolic proteins or soluble extracellular proteins. LRR: Leucine-rich repeats; LBP: LPS-binding protein; GPI: Glycosylphosphatidylinositol; LORE: Lipooligosaccharide-specific reduced elicitation; BAI1: Brain-specific angiogenesis inhibitor 1; CARD: caspase activation and recruitment domain. The figure is modified from (Kagan 2017), RightsLink permission: 4481670794410.

4 Objectives

The tested hypothesis underlying this thesis is that the early developmental temperature does not affect the future immune plasticity of zebrafish.

Consequently, this study aimed at investigating the effect of developmental temperature on the immune system of larval and adult zebrafish. It includes these three specific objectives:

1. To investigate the effect of embryonic incubation temperature and LPS-challenge temperature on the immune response of larval zebrafish under LPS challenge (**Paper I**);

2. To examine whether the embryonic incubation temperature has long-term effect on the immune transcriptome of adult zebrafish spleen (**Paper II**);

3. To investigate if there is a long-term thermal effect of embryonic incubation temperature on the spleen miRNA transcriptome, including the response to LPS (**Paper III**).

5 General discussion

Fish have high developmental plasticity during early ontogeny and tend to increase phenotypic fitness to better match their future environment (Beaman et al. 2016; Davis and Wund 2016). To understand the thermal plasticity of the immune system in larval zebrafish, we incubated zebrafish embryos at three temperatures (24, 28, and 32 °C) and assessed the mortality rate upon the LPS challenge. We chose three embryonic incubation and LPS-challenge temperature combination groups (embryonic incubation 24 °C × LPS-challenge 24 °C; embryonic incubation 24 °C × LPS-challenge 32 °C; and embryonic incubation 32 °C × LPS-challenge 24 °C) for the further transcriptomic analysis (**Paper I**).

Developmental plasticity also tends to generate persistent effects on the adult phenotypes (Koumoundouros et al. 2009; Donelson et al. 2011; Garcia de la serrana et al. 2012; Scott and Johnston 2012; Nyboer and Chapman 2017), but its lasting effect on the immune system of adult fish is poorly understood. In this thesis, we investigated the effect of thermal developmental plasticity on the splenic immune response of adult zebrafish by analyzing the mRNA (**Paper II**) and miRNA (**Paper III**) transcriptomes.

5.1 Thermal developmental plasticity of the immune system in larval zebrafish

Larval zebrafish incubated at low temperature (24 °C) during embryogenesis showed the best performance (lowest mortality) at low LPS-challenge temperature (24 °C) and poorest performance (highest mortality) at high LPS-challenge temperature (32 °C; **Paper I**). This is consistent with the viewpoint that the developmental plasticity increases the phenotypic fitness to better match the forthcoming environment (Beaman et al. 2016). However, zebrafish embryos incubated at high (32 °C) or reference temperatures (28 °C) did not show better immune performance

at the same LPS-challenge temperature (32 °C or 28 °C, respectively; **Paper I**). This could be due to the fact that the immune performance was examined during the early ontogeny (3 - 5 days post fertilization) rather than in adult stages as that described in other adaptive phenotypic plasticity studies (Nettle and Bateson 2015). Moreover, developmental temperature may have different effects on different immune elements (e.g., complement components, cytokines and phagocytosis), making the final immune performance a complex phenotype. Other non-immune physiological processes could also be affected, which potentially could affect the immune processes. Nonetheless, low embryonic incubation temperature always resulted in higher mortality rate compared to fish from reference and high embryonic incubation temperature groups under the same LPS-challenge temperature, suggesting that low embryonic incubation temperature had negative effects on the innate immunity of larval zebrafish.

Transcriptome analysis identified a number of up-regulated pro-inflammatory genes such as *il1β*, *cxcl8a*, and *prostaglandin-endoperoxide synthase 2b* (*ptgs2b*) in larval zebrafish incubated and challenged with LPS at low temperature (**Paper I**). Besides, some immune negative regulator genes, such as *nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha a* (*nfkbiαa*) and *suppressor of cytokine signaling 3b* (*socs3b*), were up-regulated, and pro-IL-1β processing gene *caspase b like* (*caspbl*) was down-regulated (**Paper I**). It is likely that both pro- and anti-inflammatory responses were elicited. Down-regulation of some antimicrobial genes, including (*lysozyme* (*lyz*), *macrophage expressed 1 tandem duplicate 2* (*mpeg1.2*), *apolipoprotein A-IV b tandem duplicate 1* (*apoa4b.1*), *cathepsin H* (*ctsh*), or *cathepsin S ortholog 2 tandem duplicate 2* (*ctss2.2*)) suggests that the effectiveness of the innate immune response could be reduced (**Paper I**). This is consistent with aforementioned suggestion that low embryonic incubation temperature had distinct effects on distinct innate immune components.

Larvae from the high embryonic incubation temperature showed a limited number of DEGs when challenged with LPS at low temperature, but a high ratio (10/33) of

them were immune-related (**Paper I**). Two mucin genes (*muc5.1*, *muc5.2*) were down-regulated, while all other immune genes such as *CCAAT/enhancer-binding protein beta* (*cebpb*), *il1b*, *cxcl8b.1*, and *ptgs2b* were up-regulated. Gene Ontogeny (GO) enrichment analysis showed similar results to those in larvae incubated and challenged with LPS at low temperature but with higher enrichment of some immune processes, such as "myeloid leukocyte activation", "leukocyte chemotaxis", "response to bacterium", and "defense response", indicating a greatly stimulated immune response (**Paper I**). The highly stimulated immune processes at high embryonic incubation temperature may contribute to the low mortality observed in this temperature group. Enriched innate immune parameters such as lysozyme activity, complement activity, respiratory burst, and neutrophil proportion have been observed in fish reared at high temperatures (Langston et al. 2002; Nikoskelainen et al. 2004; Pettersen et al. 2005), but they were examined in non-stimulated juvenile/adult fish rather than in bacterial/LPS-challenged larvae. It is unclear whether the immune transcriptome from the high embryonic incubation temperature group was similar to their reference counterparts, since the latter was not evaluated.

There was a considerably large number of DEGs in the low temperature incubation group, but only few immune-related DEGs were found when the fish were challenged with LPS at high temperature. Non immune-related DEGs included hypoxia inducible genes *myoglobin* (*mb*) and *insulin-like growth factor binding protein 1a* (*igfbp1a*), and antioxidant genes *glutathione peroxidase 1b* (*gpx1b*), *glutathione S-transferase omega 2* (*gsto2*) and *microsomal glutathione S-transferase 3b* (*mgst3b*), suggesting that hypoxia and antioxidative processes could be elicited to some extent (**Paper I**). A similar induction of *gpx* was observed in LPS-exposed zebrafish embryos (Jaja-Chimedza et al. 2012). Hypoxia can be induced by the temperature increase, and similar regulated genes and pathways were observed in Atlantic salmon exposed to high temperature and low oxygen stress (Olsvik et al. 2013). Nevertheless, the absence of significant differences in mortality among fish originating from different embryonic incubation temperatures and not exposed to

the LPS treatment suggests that the temperature increase from the low embryonic incubation temperature to the high LPS-challenge temperature has not been directly detrimental to the fish. However, we cannot exclude potential effects such as hypoxia and antioxidant processes on the immune transcriptome resulting in subsequent fish mortality.

5.2 Long-term effect of developmental temperature on the splenic immune status in adult zebrafish

We demonstrated that both low (24 °C) and high (32 °C) embryonic incubation temperatures resulted in down-regulation of a large number of immune genes in the spleen of adult zebrafish compared to reference temperature (28 °C), including those encoding cytokines, antibacterial peptides, neutrophil activity regulators, and T lymphocyte regulators (**Paper II**). In addition, high embryonic incubation temperature also decreased a number of transcripts involved in endocytosis, trafficking and lysosomal digestion processes, suggesting suppressed phagocytic capacities. The recombination-activating gene *rag1* and a high diversity of immunoglobulin genes were up-regulated in the same temperature group (**Paper II**). This is consistent with studies reporting that immunoglobulins are induced by high temperature (Hrubec et al. 1996). It is likely that these immunoglobulins are natural antibodies rather than antigen-stimulated antibodies, since all fish used in the present study have been exposed to the same aquatic environment throughout the experiment. In fact, it has been demonstrated that natural antibody activity in serum positively correlated with high temperature in Atlantic cod (Magnadottir et al. 2009). High diverse immunoglobulin transcripts can protect fish from pathogens at all times, but on the other hand, their maintenance also demands high energy allocation, restricting available energy for other physiological activities (López-Olmeda and Sánchez-Vázquez 2011).

Fish from the three temperature groups exhibited quite distinct patterns of splenic immune transcriptome to LPS challenge. The immune response to LPS challenge in the high temperature group was limited, which was probably due to some immune genes already activated before the LPS treatment, namely diverse immunoglobulin transcripts (**Paper II**). The suppressed immune transcripts in the low embryonic incubation temperature group were highly stimulated by LPS, suggesting that the suppression was reversible (**Paper II**). It seems to be a flexible strategy for fish from low embryonic incubation temperature group by shutting down a part of energy-consuming immune systems under normal conditions and immediately activating them when stimulated. However, the excessively induced inflammatory response could be harmful or even lethal for fish (Medzhitov and Horng 2009). In the reference temperature group, the immune response to LPS challenge was completely different from that employed in low or high temperature groups, primarily depending on various apolipoprotein transcripts (Apoa1a, Apoa1b, Apoeb, Apoc1, Apobb.1, Apoa2) rather than inflammatory response or diverse immunoglobulin transcripts (**Paper II**). This is consistent with the evidence that apolipoproteins are effective in neutralizing LPS (Yin et al. 2011; Beck et al. 2013). Some transcripts of inflammation-related genes, such as *Cxcl8a*, *Cxcl11.1*, *Ccl38a.4*, and *Saa* were also down-regulated in the reference temperature group. Reports have showed that fish apolipoproteins are involved in the innate immune response (Concha et al. 2004; Yang et al. 2017), and further studies demonstrated that mammalian apolipoproteins participated in the LPS-induced inflammatory response by regulating IL12 production (Ali et al. 2005; Berbée et al. 2005). Probably similar regulatory networks occurred in zebrafish from the reference temperature group in the present study.

It is known that miRNAs are involved in regulating gene expression in response to the temperature change (Campos et al. 2014a), and early developmental temperature has a long-lasting effect on miRNA expression during the later development (Bizuayehu et al. 2015). Similarly, 27 conserved and 3 novel miRNAs were found differentially expressed in fish spleen from high embryonic incubation

temperature group compared to fish kept at the constant reference temperature, and many of these miRNAs have important roles in regulating the immune system (**Paper III**). For instance, miR-217 positively regulates the germinal center response of B cells, increasing the frequency of somatic hypermutation and the generation of class-switched antibodies (De Yébenes et al. 2014). Its up-regulation probably contributed to the high diversity of immunoglobulin transcripts (**Paper II**). miR-10, miR-200a, and miR-18a function in regulating T lymphocyte differentiation (Jeker et al. 2012; Naghavian et al. 2015; Montoya et al. 2017), while let-7, miR-24, miR-194, miR-125a and miR-130b are involved in modulating NF-κB signaling pathways (Iliopoulos et al. 2009; Kim et al. 2012; Cui et al. 2016; Xie et al. 2017; Zheng et al. 2018). Over 4,300 genes were potentially targeted by 32 DE miRNAs, and some key immune processes and pathways were enriched in these target genes (**Paper III**), which is consistent with the result of mRNA transcriptome in **Paper II**. Integrative analysis of DE miRNAs (**Paper III**) and DEGs (**Paper II**) determined 31 miRNA/mRNA pairs with significant correlations, including dre-miR-125a-2-3p/june, dre-miR-122-3p/cebp1, dre-miR-122-3p/cbfb, dre-miR-733-5p/tmed8, dre-miR-7a/CU571315.1. Cebp1, Cbfb and June are important transcription factors involved in the immune response (Hai and Curran 1991; Blake et al. 2000; Lyons et al. 2001). *tmed8* encodes a transmembrane trafficking protein. CU571315.1 is an uncharacterized protein and had the strongest correlation with the expression of dir-miR-7a. These significantly expressed and correlated miRNA/mRNA pairs may have important functions in regulating the effect of developmental temperature on the splenic immune profile of adult zebrafish. Three DE miRNAs were identified in the reference temperature group after the LPS challenge, targeting 622 unique genes (**Paper III**). Enrichment analysis showed that some important immune processes (cytokine production, endocytosis, vesicle-mediated transport) and pathways (Herpes simplex infection, NIK->noncanonical NF-kB signaling, FCERI mediated NF-kB activation) were enriched by down-regulated target genes (**Paper III**), consistently with the down-regulated inflammatory response of LPS-treated fish in the reference temperature group in **Paper II**. None of these DE miRNAs was determined in the low embryonic incubation

temperature group compared to the reference temperature group, and in either low- or high temperature groups after the LPS treatment. One possibility is that some miRNAs in the high temperature group were already activated before the LPS challenge, such as the DE miRNAs aforementioned, and had non-significant expression changes responding to LPS stimulation. Alternatively, other mechanisms, such as DNA methylation, histone modification, protein modification post-translation, and endocrine hormone, could have been involved in regulating phenotypic plasticity of the immune response in these temperature groups (Beldade et al. 2011).

LPS challenge affected survival of larvae in the different temperature groups (**Paper I**). However, no mortality was observed throughout the experiment on adult zebrafish, even though a high-dosed LPS (50 mg/ml, 2 μl) was used (**Paper II, III**). Even though distinct immune transcriptomes were revealed in fish from three different temperature groups, it is difficult to determine which embryonic incubation temperature is the most beneficial for the immune system of adult fish without assessing effectiveness of the immune response.

5.3 Thermal threat in the context of climate change

The global temperature is expected to increase by 1.8 - 4.0 °C by the end of 21st century (Stocker et al, 2013), and this can influence the abundance and distribution of fish globally (Perry et al. 2005; Poloczanska et al. 2013). Some marine fish species cope with global warming by moving toward deeper or high latitude ocean areas for cooler waters (Perry et al. 2005). While freshwater fishes are restricted by terrestrial river networks, and tend to move to headwaters responding to warming temperature (Roberts et al. 2013; Turschwell et al. 2017). As a result, the same fish species could be isolated in different geographic terrains and adapted to local thermal environments independently (Narum et al. 2013). In fact, wild zebrafish live in quite different temperature ranges within a wide area (López-Olmeda and

Sánchez-Vázquez 2011). The fish used in present study have been maintained in the laboratory with stable thermal conditions (28 ± 1 °C) for generations, probably already adapted to a narrow thermal tolerance range compared to those living in the wild. We cannot exclude the possibility that a 4 °C-change caused stress for laboratory-maintained fish, which is not a threat for wild zebrafish.

In addition to moving to cooler waters, fish are able to acclimate to changing temperatures. But this capacity is restricted in stenothermal fishes, which have narrow thermal tolerance ranges and cannot cope with highly fluctuating temperatures. Stenotherms like Antarctic marine ectotherms, and warm-adapted eurytherms living near their thermal limits may be the major 'losers' from climate change (Somero 2010). It is also disadvantageous for fish with long generation-turnover time during the thermal acclimation, since the adaptive phenotype in the parental generation could be maladaptive for their offspring in the changing environment (Nettle and Bateson 2015). Zebrafish is a tropical species with a wide thermal tolerance and a short lifespan, and their wide temperature-range distribution suggests high capacity of adapting to changing temperature (López-Olmeda and Sánchez-Vázquez 2011). Other temperature-related factors probably also contribute to the phenotypic plasticity. Studies in Atlantic salmon have shown that MHC diversity increased with decreasing latitudes, which could be driven by warming temperature, increased pathogen diversity, or other unknown environmental factors (Dionne et al. 2007; Tonteri et al. 2010). Nonetheless, the knowledge about the effect of climate change on the immune system of fish is still largely unknown (Jonsson and Jonsson 2009; Crozier and Hutchings 2014). Since the adaptive developmental plasticity is one underpinning mechanism of phenotypic evolution (Davis and Wund 2016), it will be meaningful to understand the immune plasticity of fish within one generation. Our data provide clues that the embryonic incubation temperature has an effect on the developmental plasticity of immune system in larvae (**Paper I**), which is still observable in the spleen of adult zebrafish (**Paper II, III**). Based on the results obtained so far, a further study investigating the immune profiles in the offspring of the fish used in the present thesis will be

extremely interesting, in order to understand the underlying transgenerational mechanisms. Through the evaluation of the immune transcriptome and epigenetic marks of larval zebrafish in the next generation, one can ascertain whether these effects on the immune system could be inherited transgenerationally, and if epigenetic mechanisms are involved.

In aquaculture, sea-caged fish are restricted to a limited space, and may experience a wide range of temperature variations resulting from seasonal changes and extreme weathers (Brown et al. 2016). Besides, fish farms are vulnerable to thermal threats caused by direct anthropogenic activities such as warm-effluents from power plants (Sandblom et al. 2016), since they are confined. Young fish are more likely to be affected and this could have a long-term effect on their immune system.

5.4 Thermal effect on the immune function of microbiota

A variety of microorganisms reside in the gastrointestinal tract of fish, affecting nutrient acquisition, epithelial development and the host immune system (Wang et al. 2018). The composition and total viable counts of microbiota are affected by multiple factors, including water temperature. For instance, total bacterial count and *Vibrio* spp. abundance in faecal microbiota from Atlantic salmon increased with increasing water temperature, while the number of lactic acid bacteria decreased (Neuman et al. 2014). Lactic acid bacteria are probiotics protecting gastrointestinal tract of fish from invading pathogens, and their decrease could have negative influence on fish health (Gómez and Balcázar 2008; Ringø et al. 2010). In the present study, microbiota composition could have been affected by early developmental temperature, which could potentially affect the host immune performance of adult fish (Gómez and Balcázar 2008). Proliferative kidney disease is a temperature-driven parasitic disease caused by the salmonid myxozoan parasite *Tetracapsuloides bryosalmonae* (Hedrick et al. 1993); its abundance of which tends to impair microbiota homeostasis of fish and increase the risk of more bacterial infection from

surrounding water (Vasemägi et al. 2017). Besides, zebrafish intestinal alkaline phosphatase has an important role in dephosphorylating and detoxifying the lipid A of LPS generated by endogenous microbiota, and fish deficient of alkaline phosphatase are hypersensitive to LPS, which results in excessive neutrophil influx to the intestine (Bates et al. 2007). However, the role of alkaline phosphatase in detoxifying exogenous LPS and how it could be affected by temperature are currently unknown.

5.5 LPS receptors in teleost fish

To date, the LPS receptors and related signal transduction pathways in teleost fish are still a matter of debate. Zebrafish have two TLR4 homologues, but none of them can recognize LPS (Sullivan et al. 2009). Consistent with this report, no significant regulation of Tlr4 transcripts by LPS was observed in the present study. In **Paper I**, three genes including *annexin A2a* (*anxa2a*), *S100 calcium binding protein A10b* (*s100a10b*), and *lymphocyte antigen-6 epidermis* (*lye*) were up-regulated in LPS-challenged fish from all the three examined temperature groups, implying their potential roles in the LPS response and maybe even its recognition. However, none of them was up-regulated in adult zebrafish spleen after the LPS challenge (**Paper II**). Even if no other known LPS receptors or assistant proteins such as CD14, MD-2, and LPS-binding protein were significantly up-regulated, the high stimulation of immune transcriptome in fish from the low temperature group imply the existence of alternative pathways for LPS recognition and respective signal transduction (**Paper II**). Further studies are needed for elucidating the underpinning mechanisms.

6 Conclusions

The effect of embryonic incubation temperature on the immune performance of larval and adult zebrafish was investigated in the present thesis using a next-generation sequencing approach.

Low embryonic incubation temperature resulted in higher mortality of larval zebrafish under LPS challenge compared to reference and high embryonic incubation temperatures, regardless of the subsequent LPS challenge temperature. Transcriptome analysis demonstrated that zebrafish larvae incubated at a high temperature during embryogenesis regulated similar immune transcripts to their counterparts incubated at low temperature in response to LPS challenge at low temperature. In short, early developmental temperature significantly affected the immune response in larval zebrafish.

Both low and high embryonic incubation temperatures had long-term effects on the splenic immune transcriptome of adult zebrafish. Plenty of immune transcripts were suppressed in the spleen of fish from either low or high embryonic incubation temperature groups compared to their counterparts maintained at the constant reference temperature, but a high diversity of immunoglobulin transcripts was induced in fish from the high temperature group. Fish from the three different temperature groups had distinct immune transcriptomic patterns in response to LPS challenge: a large number of inflammatory transcripts were stimulated in fish from low temperature group; only limited immune transcripts were significantly regulated in fish from the high temperature group; fish kept at the constant reference temperature depended on diverse apolipoproteins. These results suggest that early developmental temperature can have a lasting effect on the immune response in the spleen of adult zebrafish.

miRNA transcriptome analysis identified some DE miRNAs in the spleen of adult zebrafish, including 32 DE miRNAs in fish from the high embryonic incubation

temperature group compared to the reference temperature group, and three DE miRNAs in fish challenged with LPS compared to unchallenged fish at the reference temperature group. Taken together, our data demonstrated that: 1) embryonic incubation temperature significantly affected the innate immune response of larval zebrafish; 2) embryonic incubation temperature had long-term effects on the splenic immune status of adult zebrafish and the response to LPS challenge; 3) miRNAs may contribute to the observed long-term effect of early developmental temperature on the immune response in the spleen of adult zebrafish. These results broadened our knowledge of thermal developmental plasticity of immune system in early fish, and their long-term effect on the immune performance of adult fish.

7 Future perspectives

Epigenetics is the study of heritable changes that do not involve DNA sequence alterations. The mechanisms of epigenetic regulation include DNA methylation, histone modification and non-coding RNA-associated gene silencing. Epigenetic modifications are involved in the regulation of early developmental environment with the later performance of fish and even in their evolution (Beldade et al. 2011; Jonsson and Jonsson 2014). Some histone modification genes including *lysine (K)-specific demethylase 2Ab* (*kdm2ab*), *Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 4a* (*cited4a*), *protein arginine methyltransferase 2* (*prmt2*), and *H1 histone family member 0* (*h1f0*) were differentially expressed in either low- or high embryonic incubation temperature groups (**Paper II**). Thus, evaluating other epigenetic modifications rather than miRNAs, such as DNA methylation and histone modifications, is a promising way of understanding their involvement in regulating the immune system of zebrafish under different temperatures.

The diversity of immunoglobulin transcripts (**Paper II**) in the spleen of fish from high embryonic incubation temperature group lead to the interesting question whether the adaptive immune system has been affected by early developmental temperature. As an important part of immune system in fish, the adaptive immune response is much more specific to recognize pathogens and has long-term memory, but it requires a long time for antibody production (Wilson 2017). Thus, a further study including more time points covering the adaptive immune response period will give a dynamic and full picture of the immune response in adult zebrafish.

Global warming is increasingly threatening fish globally, including potential epidemic outbreaks of bacterial and viral diseases. It is therefore crucial that the fish immune system is able to adapt to the increasing temperature to cope with potential pathogens. A rapid thermal acclimation of metabolic capacity to the warming ocean temperature has been observed in a tropic damselfish *Acanthochromis polyacanthus* within two generations (Donelson et al. 2012), but adaptation of fish immune

system has not been studied yet. Our data show that thermal developmental plasticity can have intragenerational effects in zebrafish, thus enabling a rapid adaptation of the immune system to the changing water temperature.

The LPS challenges performed in this thesis do not exactly reflect the real bacterial invasion process, since growth and toxicity of pathogens are also affected by the water temperature (Guijarro et al. 2015). Besides, not only infected fish can initiate immune response or use behavioral fever to limit bacterial invasion (Rakus et al. 2017b), but also pathogens are able to suppress host immune response (Steinel and Bolnick 2018) or delay their behavioral fever (Rakus et al. 2017a) to promote its own replication. During the bacterial infection, the interaction between pathogen and host results in significant regulation of transcripts from both organisms. To date, most studies focused on fish side and missed the knowledge from the pathogen side (Petit et al. 2017). Knowledge of the invading mechanism of pathogens and their relation with the host can provide a useful information for better protecting fish from the bacterial infection (Westermann et al. 2012).

The small size of larval zebrafish makes it challenging to dissect immune organs for RNA-seq. Even in adult zebrafish, the three most important immune organs: thymus, head kidney and spleen are too small for certain research approaches (Trede et al. 2004). As the spleen used in present studies contained bulk populations of immune cells including macrophages and lymphocytes, the RNA-seq result was the average transcript expression of all immune cells. ScRNA-seq is increasingly becoming a useful way to obtain transcriptome from a single cell (Wang and Navin 2015; Lafzi et al. 2018). One of the challenges in scRNA-seq is isolation of single cells, which can be performed using limiting dilution, microscope-guided capillary pipette manipulation, or laser capture microdissection (Keays et al. 2005), as well as high throughput methods such as flow-activated cell sorting (Herzenberg et al. 2002), magnetic-activated cell sorting (Schmitz et al. 1994), and microdroplet-based microfluidics methods (Mazutis et al. 2013). Based on methods used in cell isolation and library preparation, multiple sequencing approaches have been developed, including single

molecule real-time sequencing (Ardui et al. 2018), massively parallel single-cell sequencing (Jaitin et al. 2014), cell expression by linear amplification and sequencing (Hashimshony et al. 2012), and droplet-sequencing (Macosko et al. 2015). scRNA-seq is a promising tool to determine how individual immune cells or at least different immune cell types respond to temperature.

8 References

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Paper I

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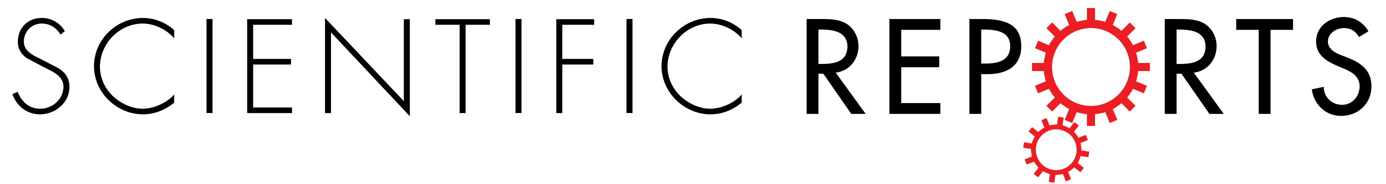

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Low incubation temperature during early development negatively affects survival and related innate immune processes in zebrafish larvae exposed to lipopolysaccharide

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In many fish species, the immune system is significantly constrained by water temperature. In spite of its critical importance in protecting the host against pathogens, little is known about the influence of embryonic incubation temperature on the innate immunity of fish larvae. Zebrafish (*Danio rerio*) embryos were incubated at 24, 28 or 32 °C until first feeding. Larvae originating from each of these three temperature regimes were further distributed into three challenge temperatures and exposed to lipopolysaccharide (LPS) in a full factorial design (3 incubation × 3 challenge temperatures). At 24 h post LPS challenge, mortality of larvae incubated at 24 °C was 1.2 to 2.6-fold higher than those kept at 28 or 32 °C, regardless of the challenge temperature. LPS challenge at 24 °C stimulated similar immune-related processes but at different levels in larvae incubated at 24 or 32 °C, concomitantly with the down-regulation of some *chemokine* and *lysozyme* transcripts in the former group. Larvae incubated at 24 °C and LPS-challenged at 32 °C exhibited a limited immune response with up-regulation of hypoxia and oxidative stress processes. *Annexin A2a*, *S100 calcium binding protein A10b* and *lymphocyte antigen-6, epidermis* were identified as promising candidates for LPS recognition and signal transduction.

In teleosts, the innate immune system is extremely important for host defence. The integumental physical barrier, which consists of skin, gill, gut and associated mucus are effective in preventing pathogens from adhering to the surface of fish[1,2]. Moreover, the mucus contains various antimicrobial substances, such as mucins, lysozymes, proteases, apolipoproteins, natural antibodies, and matrix metallopeptidase. Also, a variety of antimicrobial peptides are present, including cathelicidins, piscidins, defensins, hepcidins, and pardaxins, which not only function in pathogen cell lysis but also have roles in phagocytic chemotaxis, mast cell degranulation, and phagocytosis[3]. In most cases, the above surface barriers and associated factors are sufficient to defend the host against pathogens. If this first line of defence is breached, pathogens will encounter additional humoral immune mediators. Some mucosal antimicrobial components, such as lysozymes, proteases, complement components, also have functions in fish blood. For instance, lectins not only opsonise pathogens and prevent them from adhering to mucosal surfaces, but also activate the complement system in blood[4]. The complement system is crucial in targeting and lysing pathogens. For instance, complement component 3b (C3b) opsonises pathogens and presents them to phagocytic leukocytes, while C5b, C6, C7, C8, and C9 are able to form the membrane attack complex and lyse pathogens[5]. In addition to humoral regulators, cellular components also have key roles in the host defence. Once triggered by pathogens, leukocytes proliferate rapidly within a short time and are led by chemoattractants to infected sites. Cytokines, including interleukins (ILs), tumour necrosis factors (TNFs), chemokines, and interferons (IFNs), are also released by phagocytes; these are essential for regulation of pro- and anti-inflammatory responses[6].

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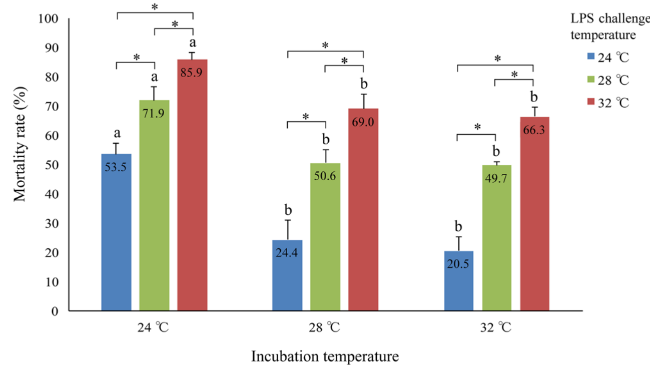

Figure 1. Mortality of larvae after LPS challenge. Mortality rates are represented as mean ± s.d. of triplicates. Significance was analysed using two-way ANOVA. Asterisks indicate the significant (p-value < 0.05) difference within the same incubation temperature group, while letters ("**a**") and ("**b**") indicate significant (p-value < 0.05) differences within the same LPS challenge temperature.

Pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) on the surface of leukocytes and other cells involved in the innate immune response. Toll-like receptors (TLRs) are one of the most important PRR families conserved among vertebrates. In vertebrates, they are phylogenetically grouped into six major families and each TLR family recognizes distinct PAMP ligand types[7]. To date, 17 TLRs have been identified in teleosts, and some paralogues have been subjected to extensive duplications[8]. Following signal transduction, the nuclear factor-κB (NF-κB) is activated and released from its inhibitor protein IκB through phosphorylation and then transferred into the nucleus where it binds to DNA to activate the transcription of pro-inflammatory cytokine genes, such as *tnfα*, *il1β*, *il6*, and *ifnγ*[9].

Teleosts lack some immune organs that are important in mammalian host defence. For instance, the bone marrow, which is the main immune organ of mammals for production of haematopoietic stem cells is absent in teleosts; its function is replaced by the pronephros and thymus[10]. Also, teleosts lack lymph nodes and germinal centres, which results in poor antibody affinity maturation and a limited immunoglobulin (Ig) repertoire[10]. Compared to the innate immune system, the adaptive immune system in fish takes longer to become fully functional[10,11]. For instance, in zebrafish (*Danio rerio*) the thymus is morphologically mature at 3 weeks post-fertilization (wpf), T cells are detectable at 4–6 wpf, and secreted Igs are measurable at 4 wpf[12]. The adaptive immune system also requires additional time to respond. For example, in Japanese eel (*Anguilla japonica*) at least two weeks are needed for the antibody production[13]. Therefore, teleosts rely heavily on the innate immune system, particularly during their early ontogeny.

As most fish are ectotherms, their body temperature changes following ambient thermal fluctuations. This is extremely challenging for the innate immune system during early stages of ontogeny in spite of their high developmental plasticity[14]. It has been demonstrated that the early thermal environment has a profound influence on various phenotypes in adults, such as muscle growth[15], swimming performance[16], reproduction[17], thermal tolerance[18], and sex determination[19]. However, little is known about the influence of environmental temperature on the developmental plasticity of the innate immune system. A few studies have focused on how temperature affects the post-larval stages of fish, but have not examined embryogenesis[20,21]. The only exception is a recent study in sea bream (*Sparus aurata*, L.) reporting the persistent thermal effect of embryonic development on the plasticity of the hypothalamus-pituitary-interrenal axis and immune function in adult fish[22].

In this study, we investigated the thermal plasticity of innate immunity in zebrafish during early development. Lipopolysaccharide (LPS) is an endotoxin from Gram-negative bacteria with well characterized immunostimulatory and inflammatory properties in fish[23]. We used LPS to mimic a bacterial challenge and the mRNA transcriptome was analysed to evaluate the global innate immune response at early larval stages.

Results

Lipopolysaccharide challenge. Mortality rates in control groups ranged from 0 to 3% (Supplementary Table S1). In LPS treatment groups, mortality rates of larvae originating from the 24 °C incubation temperature were significantly higher than those from 28 °C and 32 °C incubation temperature groups, regardless of the subsequent challenge temperatures applied (Fig. 1). For instance, at the challenge temperature of 24 °C, the mortality rate of larvae from the incubation temperature of 24 °C was 53.5%, compared to 24.4% and 20.5% in larvae from the incubation temperatures of 28 °C and 32 °C, respectively. No significant difference in mortality was observed between 32 °C and 28 °C incubation groups regardless of subsequent challenge temperatures. For larvae originating from the same incubation temperature, challenge temperatures of 24 °C and 32 °C resulted in the lowest and highest mortality rates, respectively (Fig. 1). In particular, after incubation at 24 °C, the mortality rates of larvae were 53.5% and 85.9% at the challenge temperature of 24 °C and 32 °C, respectively.

RNA sequencing and mapping. Over 376 million raw reads were obtained by RNA-seq, of which 84.1% had a quality score Q ≥ 30 (Table 1). After adapter and quality trimming, 361,234,698 clean reads were retained. Finally, 267,108,269 reads were successfully mapped to zebrafish transcriptome and genome, and 248,189,243 (92.9%) of them were uniquely mapped, including 122,554,742 read pairs (Supplementary Table S2).

	Minimum	Maximum	Total
Raw reads	11,339,354	48,009,280	376,254,382
Trimmed reads	10,897,668	46,119,584	361,234,698
≥Q30 reads	9,344,199	39,781,550	311,105,664
Mapped reads	8,183,262	34,199,926	267,108,269

Table 1. Summary of library read statistics. In total, 18 libraries, including three LPS treatment replicates and three control replicates from each of three temperature groups (Incubation 24 °C × Challenge 24 °C, Incubation 24 °C × Challenge 32 °C, Incubation 32 °C × Challenge 24 °C), were paired-end sequenced on a NextSeq 500 (Illumina).

Differentially expressed genes. A total of 605 differentially expressed genes (DEGs) (adjusted p-value < 0.05, fold change ≥ 1.5) were found in LPS-challenged larvae compared to their respective controls (Fig. 2, Supplementary Table S3). These included i) 294 DEGs (144 up-/150 down-regulated) in larvae incubated and challenged with LPS at 24 °C; ii) 33 DEGs (20 up-/13 down-regulated) in larvae incubated at 32 °C and challenged with LPS at 24 °C; and iii) 278 DEGs (190 up-/88 down-regulated) in larvae incubated at 24 °C and challenged with LPS at 32 °C. The comparison between LPS challenge temperatures revealed 207 DEGs (89 up-/118 down-regulated) specific to larvae challenged with LPS at 24 °C, 191 DEGs (135 up-/56 down-regulated) only in larvae challenged with LPS at 32 °C, and 87 DEGs (55 up-/32 down-regulated) shared by both groups (Fig. 2a). At the challenge temperature of 24 °C, there were 263 unique DEGs (124 up-/139 down-regulated) in larvae from the incubation temperature of 24 °C, 2 unique down-regulated DEGs in larvae incubated at 32 °C, and 31 common DEGs (20 up-/11 down-regulated) in both incubation temperature groups (Fig. 2b). A comparison between incubation and challenge temperatures identified 143 DEGs (51 up-/92 down-regulated) exclusively in control larvae incubated at 32 °C and challenged at 24 °C compared to larvae kept at constant 24 °C. A total of 1052 DEGs (462 up-/590 down-regulated) were only found in control larvae incubated at 24 °C and challenged with 32 °C compared to larvae maintained at 24 °C throughout experiment (Fig. 2c; Supplementary Table S6, S7).

The principal component analysis (PCA) indicated that the first principal component (PC1) explained 57% of the total variance, while PC2 explained 15% of the total variance (Fig. 3). Moreover, a higher variance of DEGs between LPS-treated larvae and control in each temperature group was observed in PC2. Hierarchical clustering and heat maps displayed different gene expression patterns in each temperature group (Fig. 4). In larvae incubated and challenged with LPS at 24 °C, two clusters were generated, and both contained some key immune-related genes (Fig. 4a). In larvae incubated at 24 °C and challenged with LPS at 32 °C, three clusters were determined, and most immune-related genes were classified into cluster III (Fig. 4b). In larvae incubated at 32 °C and challenged with LPS at 24 °C, two clusters were identified, with the transcript levels of several key immune-related genes being up-regulated in cluster II (Fig. 4c).

Immune processes regulated in response to LPS. In larvae incubated and challenged with LPS at 24 °C, a number of immune processes were enriched by up-regulated DEGs, including "response to bacterium", "myeloid leukocyte activation", "leukocyte chemotaxis", "defence response", and "response to wounding" (Fig. 5a, Table 3). In contrast, the two immune processes "response to xenobiotic stimulus" and "defence response" were enriched within the down-regulated DEGs (Fig. 5b, Table 3). In larvae incubated at 32 °C and exposed to LPS at 24 °C, similar immune processes as above were enriched at even higher values by up-regulated DEGs, including two additional processes, "regeneration", and "positive regulation of immune effector process" (Fig. 5a). No immune process was enriched by down-regulated DEGs. In larvae incubated at 24 °C and exposed to LPS at 32 °C, only three immune-related processes were stimulated compared to control, namely "response to bacterium", "response to external biotic stimulus", and "regeneration" (Fig. 5a, Table 3). In the same larvae group, two oxygen deficiency processes, "response to hypoxia" and "response to oxygen levels", were enriched (Fig. 5a, Table 3). The full Gene Ontology (GO) processes are listed in Supplementary Table S4.

KEGG pathway enrichment following LPS challenge. In larvae incubated and exposed to LPS at 24 °C, pathways such as "*Salmonella* infection", "adipocytokine signalling", "TLR signalling", "cytokine-cytokine receptor interaction", and "apoptosis" were enriched by up-regulated DEGs (Fig. 6a), while "arachidonic acid metabolism" and "fructose and mannose metabolism" were enriched by down-regulated DEGs (Fig. 6b). In larvae incubated at 24 °C and challenged with LPS at 32 °C, pathways including "steroid biosynthesis", "metabolism of xenobiotics by cytochrome P450", "fatty acid elongation", "protein processing in endoplasmic reticulum", and "phagosome" were enriched by up-regulated DEGs (Fig. 6a), while "ECM-receptor interaction", and "arachidonic acid metabolism" were enriched by down-regulated DEGs (Fig. 6b). No pathways were enriched by DEGs in larvae incubated at 32 °C and challenged with LPS at 24 °C. The full Kyoto encyclopaedia of genes and genomes (KEGG) pathways are listed in Supplementary Table S5.

Representative immune genes involved in response to LPS. Expression of several immune-related genes was significantly regulated in LPS-treated larvae compared to control in each temperature group. In larvae incubated and challenged with LPS at 24 °C, several immune-related transcripts were up-regulated with fold-changes between 1.6 and 5.3, including cytokine *il1β* and its receptors *cxcl8a*, *cxcl8b*, *tumour necrosis factor receptor superfamily, member 11b* (*tnfrsf11b*), *interleukin 13 receptor, alpha 1* (*il13rα1*), and *interleukin 6 signal transducer* (*il6st*), pro-inflammatory mediator genes *nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a* (*nfκbiαa*), *suppressor of cytokine signaling 3b* (*socs3b*), *suppression of tumorigenicity 14*

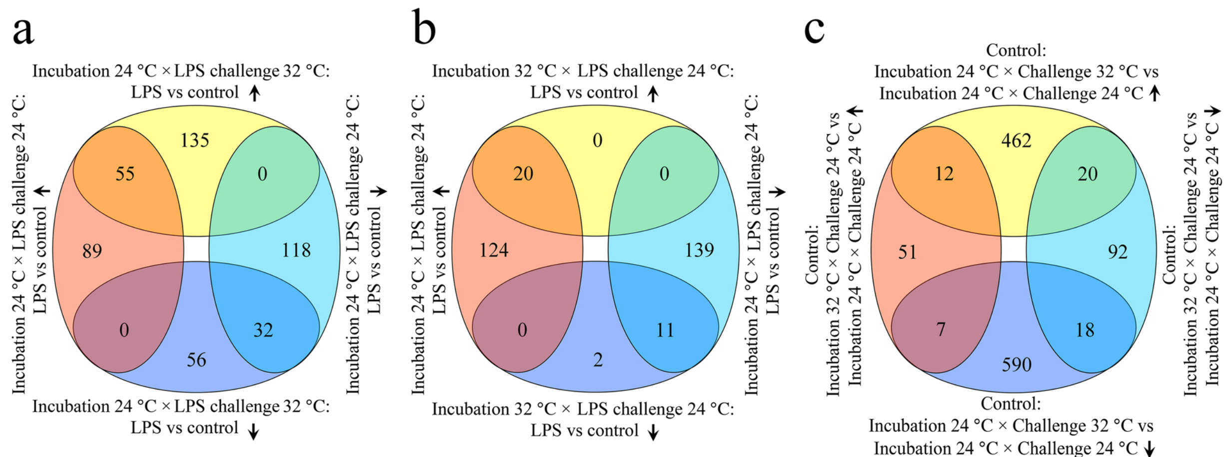

Figure 2. Venn diagram of differentially expressed genes. Comparison of DEGs (LPS-treated versus control) between larvae originating from the same incubation temperature of 24 °C but challenged with LPS at 24 °C or 32 °C (**a**), and between larvae originating from the 24 °C and 32 °C incubation temperatures, and challenged with LPS at the same temperature of 24 °C (**b**). DEGs with incubation and challenge temperatures in control larvae are also shown (**c**). Upward and downward arrows indicate up- and down-regulation, respectively.

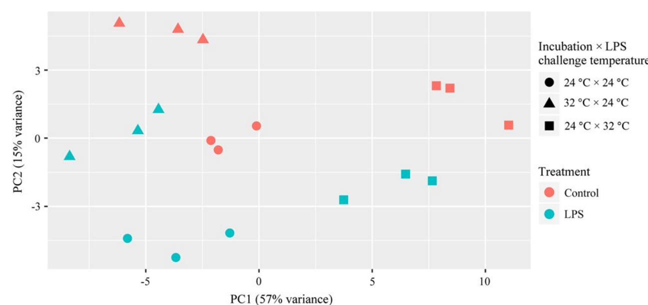

Figure 3. Principle component analyses of differentially expressed genes. PCA was performed on DEGs (LPS-treated versus control, adjusted p-value < 0.05, |fold change| ≥ 1.5) from all temperature groups. The first (PC1) and second principal components (PC2) are shown on horizontal and vertical axis, respectively.

(colon carcinoma) a (*st14a*), *fosl1a*, and *jun B proto-oncogene a* (*junba*), and chemokines *chemokine (C-C motif) ligand 34a, duplicate 4* (*ccl34a.4*) and *cxcl18b* (Table 2). Some key immune transcripts were down-regulated between 1.7 and 2.3 fold, including the antibacterial transcripts *lysozyme* (*lyz*), *macrophage expressed 1, tandem duplicate 2* (*mpeg1.2*), *cathepsin H* (*ctsh*), *cathepsin S, ortholog 2, tandem duplicate 2* (*ctss2.2*), and *apolipoprotein A-IV b, tandem duplicate 1* (*apoa4b.1*), pro-inflammatory transcripts *E74-like factor 3* (*elf3*), *leukotriene A4 hydrolase* (*lta4h*), and *caspase b, like* (*caspbl*), chemokine transcripts *ccl20a.3* and *ccl20b* (Table 2). In larvae incubated at 32 °C and challenged with LPS at 24 °C, transcript levels of some immune-related genes were up-regulated, including *il1β* (2.1-fold), *cxcl8b.1* (2.3-fold), and *CCAAT/enhancer binding protein (C/EBP), beta* (*cebpβ*) (1.8-fold) (Table 2). In larvae incubated at 24 °C and challenged with LPS at 32 °C, immune transcripts such as *immunoresponsive gene 1, like* (*irg1l*), *TIMP metallopeptidase inhibitor 2b* (*timp2b*), *leukocyte cell-derived chemotaxin 2 like* (*lect2l*), *tnfrsf11b*, *interferon regulatory factor 6* (*irf6*), and *junba* were up-regulated between 2.1- and 6.3-fold. The expression of some heat shock protein (HSP) genes such as *heat shock 60 protein 1* (*hspd1*), *hspa5*, *hsp90b1*, and antioxidant genes such as *glutathione peroxidase 1b* (*gpx1b*), *glutathione S-transferase omega 2* (*gsto2*), *microsomal glutathione S-transferase 3b* (*mgst3b*) was enhanced 1.6–2.5 fold (Table 2). In addition, transcripts such as *matrix metallopeptidase 9* (*mmp9*), *mmp13a*, *ptgs2b*, *fosl1a*, *heparin-binding EGF-like growth factor a* (*hbegfa*), *insulin-like growth factor binding protein 1a* (*igfbp1a*), *lye*, and *anxa2a* were up-regulated, whereas *mucin 5.1, oligomeric mucus/gel-forming* (*muc5.1*), and *muc5.2* were down-regulated in all three temperature groups (Fig. 4, Table 2).

Discussion

Thermal developmental plasticity of innate immunity. Animals display thermal plasticity during their embryonic development, which tends to improve their performance at that particular temperature compared to that of animals exposed to other thermal conditions[16,18]. In the present study, we have shown that the survival of LPS-challenged larvae was affected by their embryonic incubation temperature. At this ontogeny stage, the adaptive immune system of zebrafish has not yet become competent, and they rely only on innate immunity for protection against pathogens[12]. The higher mortality rate of larvae originating from 24 °C embryonic

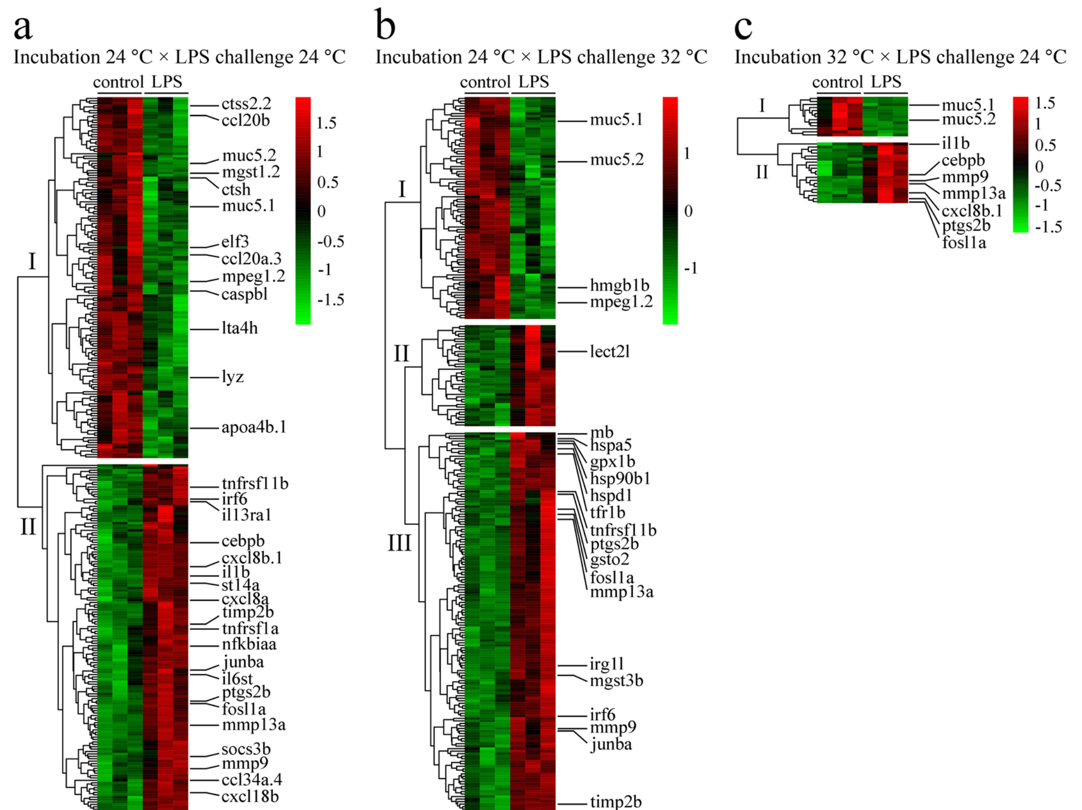

Figure 4. Hierarchical clustering and heat map of differentially expressed genes. Display based on DEGs (LPS-treated versus control, adjusted p-value < 0.05, |fold change| ≥ 1.5) for Incubation 24 °C × LPS Challenge 24 °C (**a**), Incubation 24 °C × LPS Challenge 32 °C (**b**) and Incubation 32 °C × LPS Challenge 24 °C (**c**). Log_2 transformed gene fold change is indicated by the colour scale. Hierarchical clustering groups are shown on the vertical axis. Representative genes in each temperature group are indicated.

incubation temperature, compared to that of larvae originating from 28 °C or 32 °C incubation temperatures, regardless of subsequent challenge temperatures, suggests that the innate immune response was negatively affected by the low incubation temperature (24 °C). In contrast, incubation at a high temperature (32 °C) had a negligible effect on the subsequent ability of first-feeding larvae to cope with LPS challenge. Low temperatures have been demonstrated to negatively influence the innate immune parameters, such as lysozyme activity[20], respiratory burst activity[21], opsonisation capacity[21], blood leucocyte profiles[24] and complement activity[21] in adult fish. However, this cannot be generalised to all teleosts, since enhanced innate immune parameters, including blood leucocyte percentages[20], phagocytic kidney macrophage proportion[24], and complement activity[24] have been observed in fish kept in low temperatures. This could be due to different properties of innate immune parameters or distinct sensitivities of different fish species to their environmental temperature, as described in Atlantic halibut strains[20]. It should be stressed that the above studies of thermal acclimation in fish were carried out at months or years post fertilization, when both the innate and adaptive immune systems were fully developed and functional. Therefore, they could not fully reflect the developmental plasticity of innate immunity during early ontogeny. Our study, focusing on the early life of zebrafish, found a negative effect of a low incubation temperature (24 °C) on the innate immune response of larvae to LPS challenge compared to 28 °C or 32 °C.

Effect of incubation temperature on the innate immune response to LPS. In larvae incubated and exposed to LPS at 24 °C, compared to their control in the same temperature group, the pro-inflammatory response was stimulated, as suggested by the up-regulation of expression of some pro-inflammatory genes (*il1β*, *cxcl8a*, *ptgs2b*, *cebpβ*, *fosl1a*) and processes ("response to bacterium", "myeloid leukocyte activation", "leukocyte chemotaxis", "defence response", "response to wounding"). The up-regulation of the inflammatory negative mediator transcripts *nfkbiαa*[25] and *socs3b*[26], and the down-regulation of the pro-IL-1β processing transcript *caspbl*[27], implies that the anti-inflammatory response could also be elicited. The anti-inflammatory response is a protective mechanism to quench excessive inflammatory signals, and to avoid pathophysiological consequences, such as sepsis[28]. Moreover, the down-regulation of antimicrobial transcripts (*lyz*, *mpeg1.2*, *apoa4b.1*, *ctsh*, *ctss2.2*) and immune-related processes ("response to xenobiotic stimulus", "defence response") indicates a decreased effectiveness of the innate immune response to LPS. Cationic lysozymes bind to negatively charged LPS at a stoichiometry lysozyme:LPS molar ratio of 1:3, resulting in the LPS structure transition from non-lamellar cube to the multilamella with reduced endotoxicity[29]. A significant drop of 2.3-fold in *lyz* expression can thereby weaken this neutralization effect. Apolipoproteins, a main group of high-density lipoproteins, neutralize LPS activity

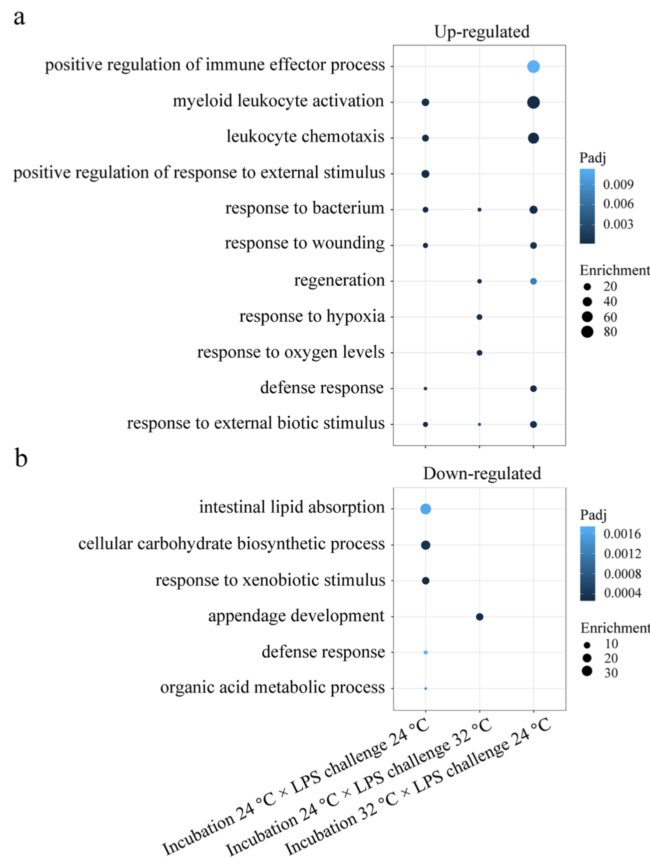

Figure 5. Representative GO processes of genes differentially regulated with LPS challenge. Up- (**a**) and down-regulated (**b**) GO processes in the different Incubation × LPS Challenge temperature groups are shown as dots, with size representing enrichment values (GeneRatio/BgRatio) and colour density reflecting their adjusted *p*-value. Significance was set at adjusted *p*-value < 0.05 (Benjamin-Hochberg method).

either by opsonizing its endotoxic lipid A domain or via blocking LPS-binding protein[30]. A drop (1.7-fold) in transcript levels of *apoa4b.1* suggests a decrease in the host capacity to neutralise endotoxic LPS. In addition, CXCL8a, CXCL8b.2, CXCL18b, and CCL34a.1 have been reported to have higher expression levels in susceptible channel catfish (*Ictalurus punctatus*) than in resistant fish when challenged with *Edwardsiella ictaluri*[31,32]. The up-regulation of *cxcl8a*, *cxcl8b.1*, *cxcl18b*, *ccl34a.4* with a fold-change between 1.7 and 2.2 may have contributed to an increased sensitivity of the larvae to LPS challenge. Both up- and down-regulated immune transcripts and processes in larvae incubated and exposed to LPS at 24 °C resulted in an intermediate mortality rate of 53.5% compared to other temperature groups (Fig. 7a).

In larvae incubated at 32 °C and exposed to LPS at 24 °C, pro-inflammatory transcripts (*il1β*, *cxcl8b.1*, *ptgs2b*, *cebpβ*, *fosl1a*) and processes ("response to bacterium", "myeloid leukocyte activation", "leukocyte chemotaxis", "defense response", "response to wounding") were up-regulated in comparison to the respective controls. The regulation trends of these immune transcripts and processes were similar to those in larvae incubated and challenged with LPS at 24 °C (Table 2, Fig. 5a), and displayed even higher enrichment values of GO processes than the latter group, implying a much stronger innate immune response to LPS. This could contribute to improve the resistance of larvae to LPS in this temperature group (incubation 32 °C × challenge 24 °C) compared to their counterparts (incubation 24 °C × challenge 24 °C). Similarly, enhanced innate immune competence was observed in fish reared at high temperatures, as manifested in serum lysozyme activity[20], complement activity[21], respiratory burst[21], neutrophil proportion[33], and IFNγ signalling pathway[34].

The change from an incubation temperature of 32 °C to the challenge temperature of 24 °C over 7 hours might have had some influence on biological processes. In common carp (*Cyprinus carpio*) that experienced cold exposure from 30 °C to either 23 °C, 17 °C or 10 °C over 1, 2, or 3 days, respectively, the expression profile of approximately 3,400 unique genes was affected[35]. To evaluate the potential effect of temperature decrease, a comparison was performed between control larvae (without LPS treatment) that experienced a temperature decrease from 32 °C to 24 °C and those kept at a constant 24 °C (Supplementary Table S6). We observed the up-regulation of transcripts of one cold-induced gene *cold inducible RNA binding protein b* (*cirbpb*) (1.6-fold) and one temperature responsive process ("response to temperature stimulus"). In particular, the nuclear receptor *nuclear receptor subfamily 1*, *group d*, *member 1* (*nr1d1*) transcripts, which code for proteins involved in both circadian and thermogenic pathways through mediation of brown adipose tissue in response to cold exposure[36], were up-regulated 3.5-fold. It has been demonstrated that the modulation of physiological metabolism occurs to mitigate the effect of temperature decrease[37]. As expected, some HSP transcripts (*hspb associated protein 1* (*hspbap1*) and *hsp70l*)

Gene Name	Description	Fold Change	Padj
Incubation 24 °C × Challenge 24 °C			
mmp13a	matrix metallopeptidase 13a	8.5	<0.001
il1β	interleukin 1, beta	5.3	<0.001
fosl1a	FOS-like antigen 1a	3.6	<0.001
lye	lymphocyte antigen-6, epidermis	3.4	<0.001
anxa2a	annexin A2a	2.9	<0.001
cxcl8a	chemokine (C-X-C motif) ligand 8a	2.2	<0.001
irf6	interferon regulatory factor 6	2.0	<0.001
cxcl8b.1	chemokine (C-X-C motif) ligand 8b, duplicate 1	2.0	<0.001
tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b	1.8	0.004
il6st	interleukin 6 signal transducer	1.6	0.002
muc5.2	mucin 5.2	−5.5	<0.001
muc5.1	mucin 5.1	−3.6	<0.001
lyz	lysozyme	−2.3	<0.001
ccl20a.3	chemokine (C-C motif) ligand 20a, duplicate 3	−2.1	<0.001
ccl20b	chemokine (C-C motif) ligand 20b	−1.9	0.002
caspbl	caspase b, like	−1.8	0.007
ctss2.2	cathepsin S, ortholog 2, tandem duplicate 2	−1.7	0.008
mpeg1.2	macrophage expressed 1, tandem duplicate 2	−1.7	0.034
apoa4b.1	apolipoprotein A-IV b, tandem duplicate 1	−1.7	0.020
lta4h	leukotriene A4 hydrolase	−1.5	0.030
Incubation 32 °C × Challenge 24 °C			
mmp9	matrix metallopeptidase 9	5.8	<0.001
mmp13a	matrix metallopeptidase 13a	4.5	<0.001
ptgs2b	prostaglandin-endoperoxide synthase 2b	3.0	<0.001
fosl1a	FOS-like antigen 1a	2.5	<0.001
lye	lymphocyte antigen-6, epidermis	2.4	<0.001
cxcl8b.1	chemokine (C-X-C motif) ligand 8b, duplicate 1	2.3	<0.001
anxa2a	annexin A2a	2.2	<0.001
il1β	interleukin 1, beta	2.1	0.002
s100a10b	S100 calcium binding protein A10b	1.9	0.002
cebpβ	CCAAT/enhancer binding protein (C/EBP), beta	1.8	0.050
muc5.2	mucin 5.2	−3.7	<0.001
muc5.1	mucin 5.1,oligomeric mucus/gel-forming	−2.3	<0.001
Incubation 24 °C × Challenge 32 °C			
irg1l	immunoresponsive gene 1, like	6.3	<0.001
mmp9	matrix metallopeptidase 9	4.8	<0.001
timp2b	TIMP metallopeptidase inhibitor 2b	4.4	<0.001
igfbp1a	insulin-like growth factor binding protein 1a	3.2	<0.001
lect2l	leukocyte cell-derived chemotaxin 2 like	2.7	0.001
lye	lymphocyte antigen-6, epidermis	2.6	<0.001
tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b	2.5	0.006
mgst3b	microsomal glutathione S-transferase 3b	2.5	<0.001
gsto2	glutathione S-transferase omega 2	2.3	<0.001
prg4b	proteoglycan 4b	2.2	0.015
irf6	interferon regulatory factor 6	2.1	0.003
gpx1b	glutathione peroxidase	2.1	0.029
mb	myoglobin	2.1	0.009
junba	jun B proto-oncogene a	2.1	0.003
itgav	integrin, alpha V	1.9	0.010
hspd1	heat shock 60 protein 1	1.7	0.006
hsp90b1	heat shock protein 90, beta (grp94), member 1	1.6	0.020
muc5.2	mucin 5.2	−3.5	<0.001
mpeg1.2	macrophage expressed 1, tandem duplicate 2	−2.0	0.008
hmgb1b	high mobility group box 1b	−1.5	0.030

Table 2. Representative genes between LPS-treated and control groups. List of selected DEGs with a fold change greater than 1.5, determined by DESeq2 (adjusted p-value < 0.05, Benjamin-Hochberg method).

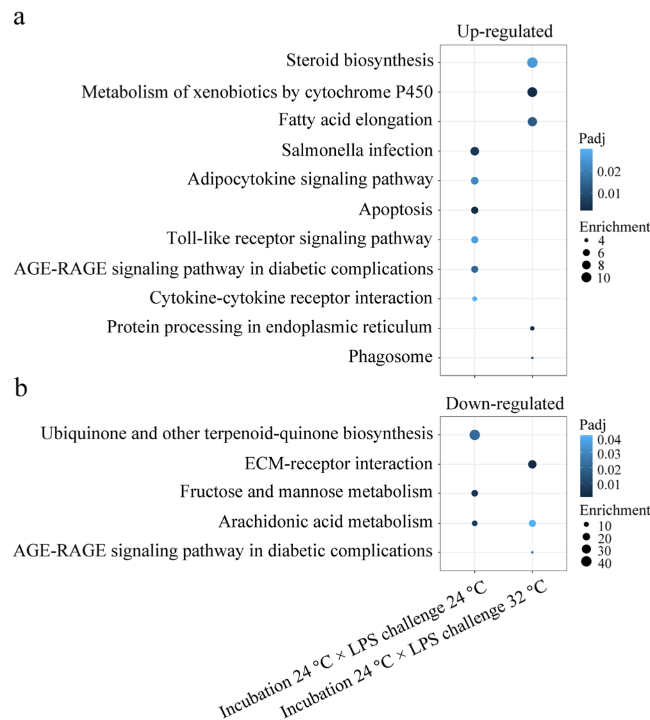

Figure 6. Selected KEGG pathways. KEGG pathways of up- (**a**) and down-regulated (**b**) DEGs were generated independently. Size is proportional to the enrichment value (GeneRatio/BgRatio), whereas colour density represents the adjusted *p*-value. Significance was set at adjusted *p*-value < 0.05 (Benjamin-Hochberg method).

were down-regulated, as well as the antioxidant gene transcripts *cytochrome P450*, *family 24*, *subfamily A*, *polypeptide 1* (*cyp24a1*) and *gpx1a*. Nonetheless, none of these processes or transcripts were significantly regulated when the LPS treatment was taken into account, indicating that the potential effect from temperature decrease on LPS-stimulated immune response was minimal. Moreover, 10 out of 33 DEGs and all (15 out of 15) GO processes were directly or indirectly related to immunity in larvae incubated at 32 °C and challenged with LPS at 24 °C, suggesting that a more effective immune response may be elicited in larvae from the 32 °C incubation temperature group compared to their counterparts incubated during embryonic development at 24 °C. These results explain the lowest mortality rate (20.5 ± 4.7%) of larvae incubated at 32 °C and challenged with LPS at 24 °C among all the temperature groups (Fig. 7c).

Effect of challenge temperature on the innate immune response to LPS. In larvae incubated at 24 °C and exposed to LPS at 32 °C, only three immune-related processes ("response to external biotic stimulus", "response to bacterium", "regeneration") were enriched, compared to their respective controls (Fig. 5a), and none of the key cytokine genes (*tnfα*, *il1β*, *il6*) was expressed at higher levels, suggesting a limited activation of the innate immune response by LPS. Nevertheless, the abundance of transcripts of some genes with important roles in inflammatory response was changed. For instance, *irg1l* transcript levels were up-regulated 6.3-fold. Its homolog gene, *Irg1*, is inducible by LPS in mouse macrophages, and encodes cis-aconitate decarboxylase to catalyse the production of the antimicrobial itaconate[38]. IRG1 is also involved in suppressing LPS-mediated sepsis and pro-inflammatory cytokine production in mouse[39]. The up-regulation of *timp2b* (4.4-fold) could either activate the pro-inflammatory NF-κB pathway in human melanoma cells, protecting cells from apoptosis[40], or exert the anti-inflammatory function by inhibiting NF-κB activity in murine microglial cells, to suppress the production of nitric oxide, TNFα, IL1β, and reactive oxygen species (ROS)[41]. Transcript levels of the cytokine gene, *high mobility group box 1b* (*hmgb1b*), which is involved in pro-inflammatory response[42], necrotic cell death[43], and sepsis[44], were down-regulated 1.5-fold. *lect2l* was up-regulated 2.7-fold; LECT2 has a neutrophil chemotactic activity specifically in the liver[45]. The protein encoded by *hspd1* (1.7-fold up-regulation) plays a critical role in regeneration and wound healing of both hair cells and caudal fins of zebrafish larvae[46]. The oxidative stress and antioxidant response of larvae were affected as well, with the induction of hypoxia processes ("response to hypoxia", "response to oxygen levels"), hypoxia inducible (*myoglobin* (*mb*), *igfbp1a*) and antioxidant genes (*gpx1b*, *gsto2*, *mgst3b*). In fact, the regulation of ROS and antioxidant activities by LPS has been demonstrated in zebrafish embryos[47,48]. Taken together, the limited inflammatory response and the induced hypoxia and oxidative stress could contribute jointly to the high mortality rate (85.9 ± 2.3%) of larvae incubated at 24 °C and challenged with LPS at 32 °C (Fig. 7b).

An increase in water temperature could lead to hypoxic conditions, which further promote the production of ROS, causing oxidative stress and affecting physiological activities. In control zebrafish larvae experiencing a temperature increase from 24 °C to 32 °C, transcripts of *oxidative stress responsive serine-rich 1* (*oser1*) and

Process	Gene-Ratio	BgRatio	Enrich-ment	Padj	Genes
Incubation 24 °C × Challenge 24 °C Up-regulated					
response to bacterium	7/84	119/14763	10.3	<0.001	*tnfrsf1α/cebpβ/mmp9/junba/ cxcl18b/il1β/junbb*
response to external biotic stimulus	8/84	186/14763	7.6	<0.001	*nfkbiαa/tnfrsf1a/cebpβ/mmp9/junba/cxcl18b/il1β/junbb*
positive regulation of response to external stimulus	3/84	21/14763	25.1	<0.001	*mmp9/cxcl18b/il6st*
myeloid leukocyte activation	3/84	24/14763	22.0	<0.001	*cxcl18b/il1β/cxcl8b.1*
leukocyte chemotaxis	5/84	49/14763	16.9	<0.001	*mmp13a/cxcl18b/cxcl8b.1/ il1β/cxcl8a*
defense response	7/84	281/14763	4.4	0.001	*ptgs2b/tnfrsf1α/mmp9/il1β/ cxcl18b/cxcl8b.1/cxcl8a*
response to wounding	9/84	200/14763	7.9	<0.001	*f3b/mmp9/sdc4/hbegfa/il6st/ f2rl1.2/cxcl8b.1/junbb/cxcl8a*
Incubation 24 °C × Challenge 24 °C Down-regulated					
response to xenobiotic stimulus	4/91	49/14763	13.2	<0.001	*foxq1a/si:ch211-117m20.5/ im:7150988/cyp1a*
defense response	7/91	281/14763	4.0	0.001	*lta4h/mpeg1.2/lyz/elf3/caspbl/ccl20b/ccl20a.3*
Incubation 32 °C × Challenge 24 °C Up-regulated					
response to bacterium	3/14	119/14763	26.6	<0.001	*cebpβ/mmp9/il1β*
myeloid leukocyte activation	2/14	107/14763	87.9	<0.001	*il1β/cxcl8b.1*
leukocyte chemotaxis	3/14	52/14763	60.8	0.001	*mmp13a/il1β/cxcl8b.1*
defense response	4/14	281/14763	15.0	<0.001	*ptgs2b/mmp9/il1β/cxcl8b.1*
response to wounding	3/14	200/14763	15.8	0.001	*mmp9/hbegfa/cxcl8b.1*
regeneration	2/14	137/14763	15.4	0.007	*mmp9/hbegfa*
Incubation 24 °C × Challenge 32 °C Up-regulated					
response to bacterium	5/129	119/14763	4.8	0.001	*lect2l/mmp9/hadhαa/irg1l/ junba*
response to external biotic stimulus	7/129	186/14763	4.3	0.004	*lect2l/cyp51/mmp9/hadhαa/ junba/irg1l/hspa5*
regeneration	7/129	137/14763	5.9	<0.001	*apoa1a/mvp/apoeb/mmp9/ hspd1/agr1/hbegfa*
response to hypoxia	4/129	45/14763	10.2	0.001	*hsp90b1/mb/igfbp1a/hspa5*
response to oxygen levels	4/129	46/14763	10.0	0.001	*hsp90b1/mb/igfbp1a/hspa5*

Table 3. Representative Gene Ontology processes regulated by LPS exposure. GO processes were enriched from DEGs by the clusterProfiler package (adjusted p-value < 0.05, Benjamin-Hochberg method). Enrichment values are defined as the ratio between GeneRatio and BgRatio. GeneRatio is the ratio of the number of genes that are annotated to a particular biological process over the size of the list of genes of interest. BgRatio is the ratio of the number of genes annotated to the biological term in the background distribution over the total number of genes in the background distribution.

reactive oxygen species modulator 1 (*romo1*) were up-regulated 1.5-fold. On the other hand, transcripts of several antioxidant genes, including *gpx1a*, *glutathione S-transferase*, *alpha tandem duplicate 1* (*gsta.1*), *gsto2*, *glutathione S-transferase pi 1* (*gstp1*), *gstp2*, *peroxiredoxin 6* (*prdx6*), and *NADPH oxidase organizer 1a* (*noxo1a*) were down-regulated 1.5–2.4 fold, as compared to larvae kept at constant 24 °C. We also noticed the up-regulation of HSP transcripts, such as *serpin peptidase inhibitor*, *clade H*, *member 1b* (*serpinh1b*), *hsp90aa1.1*, *hsp90aa1.2*, *crystallin*, *alpha A* (*cryaa*), *DnaJ heat shock protein family member A4* (*dnaja4*), *heat shock cognate 70* (*hsc70*), and the down-regulation of *antifreeze protein type IV* (*afp4*), and *cold inducible RNA binding protein a* (*cirbpa*) (Supplementary Table S7); this suggested that both hypoxia and antioxidant activities were elicited. A study in adult Atlantic salmon demonstrated that high temperature and oxygen deficiency affected quite similar genes and pathways related to heat shock and antioxidant responses[49]. Another report in two-banded seabream (*Diplodus vulgaris*), white seabream (*Diplodus sargus*), European seabass (*Dicentrarchus labrax*) and thinlip grey mullet (*Liza ramada*) showed that protective mechanisms, including the production of HSPs, and the antioxidant activity of glutathione S-transferase, catalase, and lipid peroxidation, can be enhanced to alleviate the effects from temperature increase and associated oxidative stress[50]. In our study, there were no significant differences in mortality rates of control larvae when the temperature changed from 24 °C to 32 °C, suggesting a limited effect of the temperature increase *per se*. Nevertheless, we cannot exclude a possible interaction between challenge temperature and LPS treatment.

Lipopolysaccharide signalling in zebrafish. It has been demonstrated that the regulation of immune signalling pathways in response to LPS is well conserved between teleosts and mammals[51] but alternative receptors other than TLR4 for LPS signal transduction can exist in teleosts. Some other fish-specific TLRs, such as TLR21 and TLR22, have been proposed as LPS receptor candidates[52]. However, no TLR genes showed significantly different expression in the present study. Some non-TLR receptors are also known to be involved in LPS signal transduction, such as beta-2 integrins[53], scavenger receptor[54], and C-type lectin[55]. GO analyses and InterPro annotation identified some up-regulated transcripts with potentially similar functions in zebrafish larvae exposed to LPS. *CD44 molecule a* (*cd44a*) codes for a protein with a C-type lectin-like domain and the genes *transmembrane protease*, *serine 4a* (*tmprss4a*) and *tmprss13b* encode scavenger receptors. The products of *proteoglycan 4b* (*prg4b*) and *integrin*, *alpha V* (*itgav*) genes display scavenger receptor and integrin activities, respectively. Further experimental evidence is needed to support their potential roles in sensing LPS.

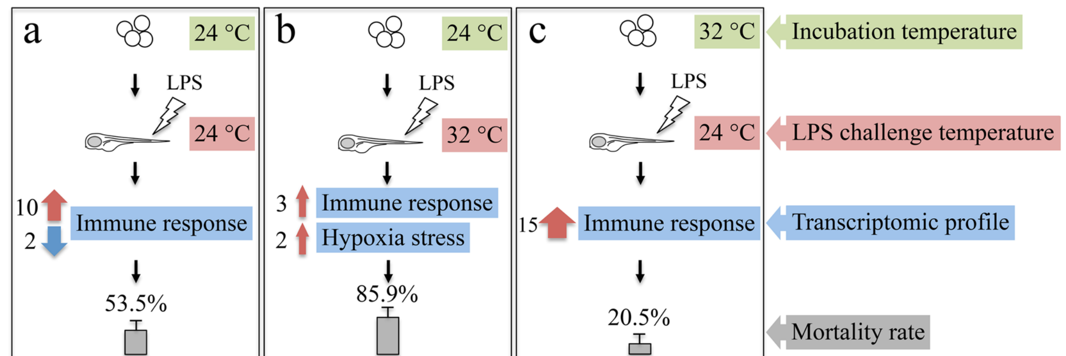

Figure 7. Diagram summarising the effect of incubation (24 °C, 32 °C) and challenge temperatures (24 °C, 32 °C) on the innate immune response of zebrafish larvae to LPS. Larvae incubated and challenged with LPS at 24 °C showed both up- (red arrow) and down-regulated (blue arrow) immune transcripts and processes following LPS challenge (**a**); larvae incubated at 24 °C followed by LPS exposure at 32 °C, displayed a weak immune response at the transcriptome level but additional hypoxia and stress transcripts were stimulated (**b**); an incubation temperature of 32 °C and subsequent LPS challenge at 24 °C elicited a strong immune response in larvae (**c**). The respective mortality rates are also indicated for each temperature group. The width of the arrows reflects the different numbers of immune- and hypoxia-related GO processes that are affected by LPS challenge in each temperature group. Only incubation temperatures 24 °C and 32 °C are shown, since the 28 °C group was not used for transcriptomic analyses.

Heat shock proteins have been implicated in LPS signal transduction. Human HSP60 contains a specific region for LPS binding[56], while murine HSP60 was able to bind to its specific receptor on the macrophage surface independent of TLR4, but its subsequent cytokine response was dependent on TLR4[57]. Another study in Chinese hamster (*Cricetulus griseus*) ovary cells revealed that HSP70 and HSP90 were involved in sensing LPS signal from CD14 and transferring to the downstream receptors[58]. Our data showed the up-regulation of *hspd1* and *hsp90b1* in larvae incubated at 24 °C and challenged with LPS at 32 °C, suggesting their possible roles in LPS signalling. Moreover, the expression of *anxa2a* and its receptor gene *s100a10b* was up-regulated in all three temperature groups. Annexin has multiple functions, including modulation of reactive oxygen species[59] and regulation of the inflammatory response triggered by TLR4[60]. Its new function as a TLR2 ligand was recently reported in mouse[61]. It is also noteworthy that the transcripts of *lye* were up-regulated between 2.4- and 3.6-fold in all three temperature groups. *Lye* is constitutively expressed in immune and epithelial cells[62], with pleiotropic functions in extracellular signal transduction, phagocyte activation, and inflammatory response[63]. The direct interaction between LPS and these genes should be investigated to ascertain their involvement in LPS recognition and signalling cascade in fish.

Conclusions

In summary, we demonstrated that both embryonic incubation and challenge temperatures affected the innate immune response to LPS in zebrafish larvae (Fig. 7). The lowest incubation temperature (24 °C) resulted in a higher mortality rate of larvae compared to the other two incubation temperatures (28 °C and 32 °C). Transcriptome analyses revealed the underlying molecular basis of this plasticity. The up-regulation of innate immune processes in response to LPS challenge was restricted in larvae originating from the lowest embryonic incubation temperature. The highest challenge temperature not only limited the immune response but also stimulated additional hypoxia and oxidative stress processes. Three genes (*anxa2a*, *s100a10b*, and *lye*), whose transcripts were up-regulated in larvae from all the temperature groups are promising receptor candidates in LPS signal transduction. These results substantially increase our understanding of the thermal plasticity of the innate immunity in zebrafish during their early development and have broader implications for fisheries and aquaculture in the context of global climate change.

Materials and Methods

Ethics statement. All animal procedures were conducted in compliance with the guidelines provided by the Norwegian Animal Research Authority (FOTS ID 8387) and approved by the Nord University (Norway) ethics committee.

Fish husbandry. Zebrafish (AB strain) were maintained in a recirculating system (Aquatic Habitats, USA) under standard husbandry conditions, including a stable temperature of 28 ± 0.5 °C and photoperiod of 12 h light: 12 h dark. Adult fish were fed SDS Small Granular diet (Special Diets Services, SDS, UK) for maintenance, and SDS 400 for conditioning prior to spawning.

Experimental design. The experimental design is illustrated in Fig. 8. Eggs were collected in the morning two hours after first light. Approximately 3,000–4,000 eggs (2- to 64-cell stage) obtained from 10 males and 20 females were pooled and then divided into three groups with an approximately equal number in each group.

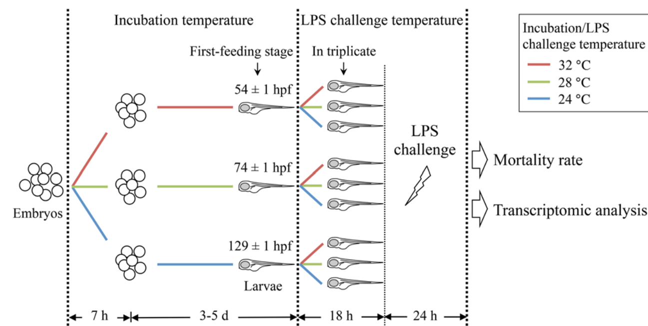

Figure 8. Experimental design. Zebrafish embryos obtained from spawning wild type fish maintained at 28 °C, were randomly assigned to three groups and incubated at 24 °C, 28 °C, or 32 °C (incubation temperature) throughout embryonic development. At the first-feeding stage, larvae from each incubation temperature group were divided into three new groups, followed by a temperature change to either of 24 °C, 28 °C or 32 °C (LPS challenge temperature) over 7 hours. At 18 h post the first-feeding stage, the LPS challenge was performed in all 9 temperature groups (3 incubation temperatures × 3 challenge temperatures). Mortality was evaluated at 24 h post LPS challenge. Groups exhibiting significantly different mortality rates were chosen for further transcriptomic analyses.

The temperatures of three groups were adjusted to 24 °C, 28 °C, and 32 °C, respectively, at a rate of 0.6–0.8 °C/h. Eggs were incubated in sterile E3 medium containing 0.1 mg/L methylene blue (Sigma-Aldrich, USA) until the first-feeding stage. This standard ontogeny stage is defined as the point when the swim bladder is inflated, the mouth is protruding and larvae start to actively seek food[64]. One-third of the medium was changed daily and larvae were not fed throughout the experiment. When 75% reached the first-feeding stage (129 ± 1 hpf at 24 °C, 74 ± 1 at 28 °C, 54 ± 1 at 32 °C; Supplementary Table S1, Supplementary Fig. S1c), larvae from each incubation temperature group were further divided into the three challenge temperature groups (24 °C, 28 °C, 32 °C), and the temperature adjustments were performed as above. As shown in Supplementary Fig. S2, there were no significant differences in body length with incubation temperature (4.1 ± 0.2 mm at 24 °C, 4.0 ± 0.1 mm at 28 °C, and 4.1 ± 0.2 mm at 32 °C; mean ± s.d., n = 10). A full factorial design of three incubation temperatures and three challenge temperatures yielded nine temperature combinations. A total of 18 beakers (nine for LPS challenge and nine for control) were used. After 18 h, some larvae from each incubation × challenge temperature group were immersed in distilled water containing 10 µg/L LPS from *Pseudomonas aeruginosa* 10 (Sigma-Aldrich, USA). LPS was prepared as a stock solution at 10 mg/L in standard phosphate-buffered saline (Sigma-Aldrich, USA). The remaining individuals (controls) were immersed in distilled water containing the same dose of phosphate-buffered saline (200 µL). Larvae were kept in open 500 mL beakers immersed in fish tanks at 24 °C, 28 °C or 32 °C (challenge temperature) at a density of 149 ± 32 larva per 200 mL (n = 54, Supplementary Table S1). At the start (0 h) and 24 h post LPS challenge, mortality rates of LPS-treated and control larvae were determined in triplicate. Significant differences were evaluated by two-way analysis of variance (ANOVA) and LSD *post hoc* test with SPSS Statistics (v21.0.0.0, IBM). The ANOVA assumptions of normality and equal variance of the data were verified by Kolmogorov-Smirnov test and by Levene's test, respectively. Statistical significance was determined at p-value < 0.05. Mortality rates were presented as mean ± standard deviation (s.d.). The experiment was repeated three times using randomly selected broodstock fish from the same laboratory population.

At 24 h post LPS challenge, three LPS challenge replicates and three control replicates from each of the three incubation × challenge temperature groups (incubation 24 °C × challenge 24 °C, incubation 24 °C × challenge 32 °C, incubation 32 °C × challenge 24 °C) were chosen for further transcriptomic analyses. All three incubation × challenge temperature groups showed significantly different mortality rates following LPS challenge (Fig. 1). Larvae were euthanized with 300 mg/L tricaine methanesulfonate (MS-222; Sigma-Aldrich, USA), snap-frozen in liquid nitrogen and stored at −80 °C until use. To obtain sufficient total RNA for transcriptome sequencing, each replicate was a pool of five larvae.

Total RNA isolation, library preparation and mRNA sequencing. Samples were homogenized at 6,500 rpm for 2 × 20 s in a Precellys 24 homogenizer (Bertin Instruments, France). Total RNA was extracted from whole larvae following the QIAzol protocol (Qiagen, Germany). RNA concentration, purity and quality were determined using the NanoDrop 1000 (Thermo Scientific, USA) and the TapeStation 2200 (Agilent Technologies, USA).

TruSeq libraries were prepared from total RNA according to the manufacturer's protocol (Illumina, USA). After purification with oligo-dT beads, mRNAs were washed and fragmented into an average length of 508–541 base pairs. The first strand of complementary DNA was synthesized with random hexamer primers (Illumina, USA), while the second strand was synthesized by Second Strand Master Mix (Illumina, USA). All 18 libraries were barcoded and normalized with the KAPA library quantification kit (Kapa Biosystems, USA). The pooled libraries were then denatured according to the NextSeq System Denature and Dilute Libraries Guide (Illumina, USA) and loaded at 11 pM on a NextSeq 500 reagent cartridge (Illumina, USA) for 150 cycle, paired-end sequencing at the Nord University genomics platform (Norway).

Bioinformatics analyses. Raw RNA-seq data were converted to FASTQ format with bcl2fastq2 (v2.17, Illumina), followed by quality control using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and adapter removal using cutadapt (http://cutadapt.readthedocs.io/en/stable/guide.html) with the parameters: -q 30,25 --quality-base = 33 --trim-n -m 20. Clean reads were mapped to the zebrafish transcriptome (GRCz10.86.chr.gtf) and genome (GRCz10.dna.toplevel.fa) from Ensembl (http://www.ensembl.org) using TopHat2[65] with parameters: -r 100 --mate-std-dev 100. Mapped reads were counted against the reference transcriptome (GRCz10.86.chr.gtf) by HTSeq-count (http://htseq.readthedocs.io/en/release_0.9.1/), and further used for differential expression analyses by DESeq2[66] to compare LPS-treated versus control groups. DEGs were determined by DESeq2 with an adjusted p-value < 0.05 (Benjamin-Hochberg method). DEGs with a |fold change| ≥ 1.5 were subjected to Gene Ontology (GO) biological process and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses by clusterProfiler[67]. Graphical representation was achieved using ggplot2 and pheatmap R packages.

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Author Contributions
J.M.O.F., I.B. and Q.Z. designed the experiment. Q.Z. performed the experiments and data analyses, and drafted the manuscript. M.K. and Q.Z. conducted the RNA sequencing. J.M.O.F. and I.B. revised the manuscript.

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Paper II

Embryonic incubation temperature has a long-term effect on the immune transcriptome and its response to lipopolysaccharide in the spleen of adult zebrafish *Danio rerio*

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11 # Abstract

12 Most teleosts are ectotherms and their immune system is sensitive to ambient thermal
13 environment. Thermal plasticity during embryogenesis can have a long-term effect in
14 adult phenotypes and behavior. However, little is known about the effects of
15 temperature during early ontogeny on the immune system of an adult fish. Herein, we
16 incubated zebrafish embryos at three temperatures (24 °C, 28 °C and 32 °C) during
17 embryogenesis, and then reared them in a common garden at 28 °C from the first-
18 feeding stage onwards. At 100 days post fertilization, fish from each temperature
19 groups were intraperitoneally injected with lipopolysaccharide (LPS) and kept for
20 another 12 h. The spleen transcriptome was analyzed with focus on immune-related
21 transcripts. We found that embryonic incubation temperatures differentially affected
22 the repertoire of immune transcripts in adult zebrafish. Both 24 °C and 32 °C
23 embryonic incubation temperatures reduced the level of cytokine transcripts (*cxcl8a*,
24 *cxcl11.1*, *ccl20a.3*, *tnfa*, *tnfb*, *il12ba*, *il12bb*, *saa*), as well as transcripts involved in
25 neutrophil- (*serpinb1l2*, *ncf1*, *ncf4*) and T-cell activity (*sema4ab*, *crtam*, *alcama*,
26 *tagapa/b*) compared to the 28 °C group. Moreover, 32 °C also attenuated transcripts
27 associated with the endosome-lysosome system, but promoted transcripts encoding
28 diverse immunoglobulins. We also found that embryonic incubation temperature
29 differentially affected the immune response to LPS challenge in adult fish. Zebrafish
30 kept at 28 °C had a higher expression of various apolipoprotein genes in the spleen but
31 reduced levels of inflammatory transcripts after LPS treatment compared to untreated
32 fish in the same temperature group. Zebrafish from the 24 °C embryonic incubation
33 temperature group responded to LPS with significantly increased transcripts involved
34 in inflammation, granulocyte/macrophage differentiation, neutrophil respiratory burst,
35 antibacterial, heat shock proteins, T lymphocyte activation, and endosome-lysosome
36 system, while the 32 °C group showed limited response to LPS, with only few up-
37 regulated immune genes. In conclusion, this study demonstrated that embryonic
38 temperature has a long-term effect on immune transcriptome and its response to LPS
39 challenge in the spleen of adult zebrafish.

40

Abbreviations

LPS: lipopolysaccharide; DEG: differentially expressed gene; dpf: day post-fertilization; i.p.: intraperitoneally; hpi: hour post-injection; GO: gene ontology; KEGG: Kyoto encyclopaedia of genes and genomes; PCA: principal component analysis; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor; PAMP: Pathogen-associated molecular pattern; PPR: pattern recognition receptor; TLR: Toll-like receptor; HSP: heat shock protein; ROS: reactive oxygen species; RIN: RNA integrity number

Key words

Thermal plasticity, embryonic development, immunity, teleost

52 ## Introduction

53 Jawed fishes are the most ancient vertebrates that possess both innate and adaptive
54 immune systems [1]. The mucosal barrier, cellular (macrophages, neutrophils, non-
55 specific cytotoxic cells) and humoral components (complement components,
56 lysozymes, proteases, cytokines, chemokines, natural antibodies) form the innate
57 immune defense line [2], while thymus, pronephros and spleen are pivotal immune
58 organs for the differentiation and maturation of adaptive immune cells [3]. Pathogens
59 are recognized through their pathogen-associated molecular patterns (PAMPs) by
60 pattern-recognition receptors (PRRs) of host immune cells [4]. The central PRRs in
61 teleosts are Toll-like receptors (TLRs) [5], while the most PAMP of Gram-negative
62 bacteria is lipopolysaccharide (LPS) [6].

63 Serum LPS-binding protein is able to either deliver LPS to TLR4/MD-2 receptor complex
64 on the monocyte/macrophage membrane to initiate intracellular NF-κB pathway and
65 inflammatory response [7], or transfer LPS to high-density lipoproteins (HDLs) for the
66 final detoxification in a dose-dependent manner [8]. Alternatively, LPS-binding protein
67 integrates into cell membranes and facilities LPS internalization to cytoplasmic
68 receptors [9]. In addition, other receptors such as scavenger receptor, complement
69 receptors, Fc receptors, are involved in recognizing and internalizing LPS, through
70 which LPS is trafficked to endosome, fused to lysosome and detoxified by hydrolase
71 [10].

72 Fluctuating temperature, exacerbated by climate change and anthropogenic activities,
73 is becoming a challenging factor for teleosts, since most of them are ectotherms [11].
74 This affects a suite of *in vivo* physiological processes in fish such as *heat shock protein*
75 (*hsp70*) expression [12] and metabolic enzyme activity [13], especially during the early
76 ontogeny when the developmental plasticity is still high [14]. In particular, the immune
77 system is affected by varying temperatures during the early development. For instance,
78 young zebrafish reared at 15 °C from 2 to 29 days post-fertilization (dpf) showed
79 distinct expression patterns of pro-inflammatory and antiviral genes in response to
80 either viral hemorrhagic septicemia or poly I:C compared to their counterparts
81 maintained at 28 °C [15]. Moreover, even a short-term (3-5 d) embryonic incubation
82 under different thermal conditions affected the survival of larval zebrafish and
83 differential regulation of immune-related genes in response to LPS challenge [16]. A
84 recent study in sea bream (*Sparus aurata,* L.) suggested that the thermal experience
85 during either embryonic or larval ontogeny affected the number of
86 metanomacrophage centers and the expression of a phagocytic gene, *dopachrome*
87 *tautomerase* (*dct*) in the pronephros under acute confinement stress in adult fish [17].
88 Nonetheless, it is still largely unknown to date whether thermal conditions during the

89 early ontogeny affect the global immune transcriptome and its responsiveness to
90 pathogen challenge in the further development.

91 The teleost spleen is an important secondary immune organ that contains abundant
92 mature myeloid cells and B-/T-lymphocytes [18]. In the present study, we incubated
93 zebrafish embryos under low (24 °C), high (32 °C) or control (28 °C) temperatures
94 during the embryogenesis. At the first-feeding stage, low and high temperature-
95 incubated larvae were transferred to the control temperature and reared in a common
96 garden for 100 days to investigate the long-term effect of embryonic incubation
97 temperature on the spleen transcriptome before and after LPS challenge.

98

Materials and methods

Ethics statement

All animal procedures were conducted in compliance with the guidelines provided by the Norwegian Animal Research Authority (FOTS ID 13900) and approved by the Nord University (Norway) ethics committee.

Experimental design and fish maintenance

The experimental design is shown in Fig. 1. Zebrafish (AB line) were kept in a recirculating aquatic system (Aquatic Habitats, USA) at 28 ± 0.5 °C with photoperiod of 12 h light: 12 h dark. Ten males and 20 females were randomly chosen for breeding. Cleavage stage embryos were collected in the morning. The embryos were divided into three groups (~100 per group) that were incubated at 24 ± 0.5 °C (low temperature), 28 ± 0.5 °C (reference temperature), and 32 ± 0.5 °C (high temperature), respectively. The water temperature was adjusted as previously reported [16]. During the first 24 h after collection, embryos were kept in water containing 0.1 mg/L methylene blue (Sigma-Aldrich, USA) to prevent fungus growth. When larvae reached the first-feeding stage, the temperature in aquaria hosting low and high temperature groups was gradually adjusted to 28 ± 0.5 °C. Larvae and juvenile were maintained in static water. At one month post-fertilization, juvenile were transferred to the recirculating system with slow-flowing water. At 100 days post-fertilization, 7 females from each embryonic incubation temperature groups were intraperitoneally (i.p.) injected with 2 µl of 50 mg/ml LPS from *Pseudomonas aeruginosa* 10 (Sigma-Aldrich, USA); another 7 female replicates per group were i.p. injected with 2 µl phosphate-buffered saline (Sigma-Aldrich, USA) as control. During all the experiment, fish were fed SDS (Special Diets Services, SDS, UK) 100, 200, 300, and 400 sequentially according to their developmental stages.

Sampling and RNA extraction

At 12 hours post-injection (hpi), the fish were euthanized with 300 mg/L tricaine methanesulfonate (MS-222; Sigma-Aldrich, USA). The spleen organ was dissected, snap-frozen in liquid nitrogen, and stored at -80 °C. Total RNA was extracted using the PicoPure RNA Isolation kit (ThermoFisher Scientific, USA) with some modifications on protocol. In brief, the spleen was immersed in 100 µl extraction buffer, mashed using a 1 ml pipette tip, and the suspension was re-pipetted several times to dissociate the cells completely. The homogenate was incubated at 42 °C for 30 min and then centrifuged at 3,000 × g for 2 min. The supernatant containing RNA was transferred to

a new microcentrifuge tube, mixed thoroughly with 100 µl 70% ethanol, and bound to the preconditioned purification column by centrifugation. The RNA was sequentially washed with wash buffer 1 and wash buffer 2, and then dissolved in 30 µl elution buffer. RNA quality and concentration were determined using the High Sensitivity RNA ScreenTapes on a TapeStation 2200 (Agilent Technologies, USA).

Libraries preparation and RNA sequencing

Five LPS treatment replicates and five controls from each embryonic incubation temperature group (n = 30 in total) were used for building mRNA libraries. Fifty ng of high quality total RNA (RIN > 7.0) from each replicate was purified with Oligo(dT) beads, fragmented to ~200 nucleotides by incubating at 94 °C for 15 min, and reverse transcribed into the first and second strand complement DNAs (cDNAs), following the manual of the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB, USA). The double-strand cDNA was end-repaired, ligated with universal adapters on the 5' end and indexed adapters on the 3' end. The ligated cDNAs were amplified on a PCR thermal cycler for 14 cycles (98 °C for 30 s, 1 cycle; 98 °C for 10 s, 65 °C for 75 s, 14 cycles; 65 °C for 5 min, 1 cycle), followed by purification with SPRIselect beads. The quality and quantity of mRNA libraries were assessed using High Sensitivity D1000 ScreenTapes on a TapeStation 2200 (Agilent Technologies, USA). The libraries were arranged on two flow cells, with a balanced representation of all groups in order to minimize potential sequencing variation. The libraries were sequenced on a NextSeq 500 platform (Illumina, USA) with a high output flow cell (single-end, 75 bp length) at our sequencing facility (Nord University, Norway).

Bioinformatics analyses and statistics

Raw mRNA reads were converted to fastq format with bcl2fastq2 (v2.17, Illumina), followed by quality control using FastQC [19], adapter removal using cutadapt [20], and low-quality read filtering with the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Clean reads were aligned to the latest version of zebrafish transcriptome (GRCz11.92) and genome (GRCz11) [21], and were counted using STAR [22]. The read counts from all 30 samples were merged into a single matrix file. Differential expression analysis was performed using DESeq2 [23], and transcripts with an adjusted p-value < 0.05 (Benjamin-Hochberg method) and a |fold change| ≥ 1.5 were further subjected to Gene Ontology (GO) enrichment, Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Reactome pathways using the DAVID online tool [24], with the significance threshold of an EASE score (modified Fisher Exact p-value) < 0.05. Enriched GO biological processes were collected at level 3

172 and “DIRECT” (default by DAVID), and redundant and too general terms were
173 removed. ggplot2, VennDiagram, and pheatmap R packages were used for graphical
174 representation of the data.

175

176 # Results

177 *Library characterization and overview of the spleen transcriptome in*
178 *zebrafish*

179 Over 930 million raw reads were obtained by RNA-seq, of which more than 892 million
180 passed quality control, and 96.9% were mapped to zebrafish transcriptome or genome
181 (Table 1, Supplementary file 1). One control library from the 24 °C embryonic
182 incubation temperature group was discarded due to high variance and low correlation
183 with the other four replicates (Supplementary file 2). All remaining 29 libraries were
184 included in the downstream analysis. Among the top 20 most abundant transcripts,
185 five encoded haemoglobin proteins, three were cytochrome c oxidase subunits, and
186 one encoded a heavy polypeptide of ferritin (Table 2). The raw data were deposited at
187 Gene Expression Omnibus under the accession GSE121163.

188

189 *Effect of embryonic incubation temperature on immune homeostasis*

190 To determine the effect of embryonic incubation temperature on the immune status
191 of adult zebrafish spleen, we compared both 24 °C and 32 °C incubation groups to the
192 28 °C control group. In total, 251 differentially expressed genes (DEGs; 71 up-/180
193 down-regulated) and 660 DEGs (385 up-/275 down-regulated) were identified,
194 respectively, in 24 °C versus 28 °C and 32 °C versus 28 °C pairwise comparisons
195 (Fig. 2A, B, Supplementary file 3). The unique DEGs explained 50% of the variance
196 between the three embryonic incubation temperatures in the first primary
197 component, while 14% of the variance was explained by the second primary
198 component (Fig. 2C). All the 796 unique DEGs were classified into four clusters based
199 on their expression profiles in the three temperature groups. Some DEGs were
200 common to both comparisons, including a number of immune-related genes. For
201 example, in the cluster 3 the transcripts of some genes were up-regulated in both
202 comparisons, while in the clusters 1 and 2 a large number of cytokine genes and other
203 immune-related genes had lower expression levels in both 24 °C and 32 °C incubation
204 groups, as compared to the 28 °C group (Fig. 2D). Most of DEGs were associated
205 uniquely with one of the comparisons only (Fig. 2B). For instance, some components of
206 *nuclear factor of kappa light polypeptide gene enhancer in B-cells* (*nfkb*) signaling
207 pathway were significantly down-regulated in the 24 °C group but not in the 32 °C
208 group. In the 32 °C group, plenty of immune genes were specifically up-regulated,
209 including those encoding immunoglobulins, complement components, and TLRs, while
210 some transcripts involved in regulating cytokines, neutrophil differentiation and
211 respiratory burst, endocytosis and endosomal trafficking, proteasome, and lysosome

212 were exclusively down-regulated in 32 °C group (Fig. 2D, Table 3, Supplementary
213 file 3).

214 Enriched GO biological processes, KEGG and Reactome pathways were examined
215 based on DEGs obtained from the above comparisons. In samples from the 24 °C
216 embryonic incubation group, only few immune processes were enriched in up-
217 regulated transcripts, while multiple inflammatory processes and pathways were
218 enriched in down-regulated transcripts (Fig. 3A, Supplementary file 4). In 32 °C
219 incubation group samples, a number of innate and adaptive immune processes were
220 overrepresented by up-regulated DEGs, while some pro-inflammatory processes were
221 overrepresented by down-regulated DEGs (Fig. 3). Besides, up-regulated DEGs
222 enriched pathways of "mTOR signaling" and "p53 signaling", while down-regulated
223 DEGs enriched immune pathways related to "Phagosome", "Cytokine-cytokine
224 receptor interaction", "Toll-like receptor signaling" (Fig. 3B, Supplementary file 4).

225

226 *Differentially expressed genes in response to LPS challenge*

227 A total of 567 DEGs (271 up-/296 down-regulated), 140 DEGs (80 up-/60 down-
228 regulated) and 49 DEGs (11 up-/38 down-regulated) were identified in LPS-treated fish
229 compared to their controls in 24, 28 and 32 °C groups, respectively (Fig. 4A). Generally,
230 more immune-related transcripts were induced in the 24 °C group than in 28 °C group.
231 In contrast, samples from the 32 °C embryonic incubation temperature showed limited
232 induction of immune transcripts as compared to the reference temperature group
233 (Fig. 4B, Supplementary file 5).

234 Transcriptome responsiveness to LPS treatment was predominantly specific to the
235 embryonic incubation temperature, with very few genes showing altered expression in
236 two or three temperature groups. Only one gene, *period circadian clock 2* (*per2*), had
237 significantly increased expression in all the three groups. Besides, *cryptochrome*
238 *circadian clock 1aa* (*cry1aa*) had increased expression in both 24 °C and 28 °C groups,
239 and *cryptochrome circadian clock 5* (*cry5*) had increased expression in 28 °C and 32 °C
240 groups, while the long intergenic noncoding RNA gene *FP102783.1* had decreased
241 transcript levels in 28 °C and 32 °C groups (Fig. 5A, B).

242 Other DEGs were temperature group-specific. For instance, 269 DEGs were up-
243 regulated in the 24 °C group, including genes related to antibacterial functions,
244 endosome formation and vesicular transport, lysosomal components,
245 granulocyte/macrophage differentiation, neutrophil respiratory burst, pro- and anti-
246 inflammatory responses (Fig. 4B, Table 4). They enriched immune response,
247 phagocytosis processes and related pathways (Fig. 5C, E, Supplementary file 6).

248 Transcripts of 296 DEGs were down-regulated in the same group (24 °C), including
249 some autophagy-promoting genes (Table 4). As a result, they enriched autophagy and
250 cell division processes and pathways (Fig. 5D, F, Supplementary file 6).

251 In the 28 °C group, 77 DEGs were up-regulated in response to LPS treatment, including
252 diverse apolipoprotein and immunoglobulin genes (Fig. 4B, Table 4), which enriched
253 the lipoprotein metabolic and antigen presentation processes (Fig. 5C). Transcript
254 levels of 59 DEGs were down-regulated in the same group in response to LPS including
255 some cytokine genes (Table 4), which enriched inflammatory processes and cytokine
256 signaling pathways (Fig. 5D, F, Supplementary file 6). In the 32 °C embryonic
257 temperature group, only 9 DEGs had increased expression in response to LPS and only
258 37 DEGs showed decreased expression. The down-regulated DEGs enriched
259 complement activation and other humoral immune-related processes (Table 4, Fig. 5D,
260 Supplementary file 6).

261

262 ## Discussion

263 ### *The effect of embryonic temperature on the spleen transcriptome*

264 The present study demonstrated that a short-term (3 - 5 d) exposure to different
265 thermal conditions during embryonic development affected future spleen
266 transcriptome in young adult fish (Fig 6). A large number of cytokine and negative
267 regulator genes (*cxcl8a*, *cxcl11.1*, *ccl20a.3*, *ccl35.1/ccl35.2*, *il12ba*, *il12bb*, *tnfa*, *tnfb*,
268 *saa*, *socs1a*), superoxide-producing genes (*ncf1*, *ncf4*) and T lymphocyte activity genes
269 (*tagapa/tagapb*, *sema4ab*, *crtam*, *alcama*) were expressed at lower levels in zebrafish
270 spleen from the 24 and 32 °C embryonic incubation temperature groups compared to
271 their counterparts kept at the constant 28 °C. Cytokines have a critical role in initiating
272 functional leukocyte and inflammatory responses, but also they are important in
273 regulating hematopoiesis and maintaining immune homeostasis [25,26]. Besides, the
274 most down-regulated transcript in both groups (*serpinb1l2,* fold change: -11.9 in
275 24 °C, -10.4 in 32 °C) encodes a serpin peptidase inhibitor that specifically neutralizes
276 over-expressed proteinase of neutrophils [27]. Thus, it is likely that low and high
277 embryonic incubation temperatures had a long-term effect on modulating immune
278 gene expression, resulting in lower levels of cytokine transcripts and other immune
279 gene expressions compared to fish kept at 28 °C (Fig. 6).

280 Expression of a high diversity of immunoglobulin genes (*cd74a*, *cd74b*, *ighv1-4*, *ighd*,
281 *igl3v5*, *igl1c3*, *igl4v9*, *igsf9a*, *igic1s1*) was up-regulated in the 32 °C incubation group
282 compared to the 28 °C group. One explanation is that the up-regulated transcript of
283 *rag1*, and MHC class II transactivator gene *ciita* contributed to generate diverse
284 immunoglobulins. These immunoglobulin genes are likely to be natural antibodies,
285 since all fish were maintained in the same recirculating aquatic system and exposed to
286 the same antigens but these immunoglobulin genes were only significantly up-
287 regulated in fish from the 32 °C embryonic incubation temperature group. Natural
288 antibodies have an important role in immune defense [2,28], and their activities have
289 been positively associated with the high rearing-temperature in Atlantic cod (*Gadus*
290 *morhua* L.) [29]. The down-regulation of transcripts related to endocytosis, vesicular
291 transport, lysosomal acidification (*rab5ab*, *rab7*, *pikfyve*, *atp6ap1b*, *atp6v1ba*,
292 *atp6v1ab*, *atp6v0b*, *atp6v1aa*, *atp6v1c1b*, *atp6v1h*, *atp6ap2*, *atp6v0d1*) and
293 proteasome subunits (*psmc3*, *psmc4*, *psmc6*) suggests that the intracellular transport
294 and protein degradation systems were affected (Fig. 6). In zebrafish, the innate
295 immune system starts to function as early as 1 dpf, while the thymus, kidney and
296 spleen primordia appear by 2-4 dpf at 28 °C [18,30]. Our results suggest that during
297 this time window, the development of the spleen was affected by the embryonic
298 incubation temperature and that this effect was still noticeable in the adult fish.

299

300 *Embryonic incubation temperature induces different molecular responses*
301 *to LPS challenge*

302 The present study also showed that zebrafish spleen from different embryonic
303 incubation temperatures had distinct responses to LPS challenge. In samples from the
304 28 °C incubation group, a number of apolipoprotein transcripts (*apoa1a, apoa1b,*
305 *apoeb, apoc1, apobb.1, apoa2*) was significantly elevated at 12 hpi, while inflammatory
306 transcripts (*cxcl8a*, *cxcl11.1*, *ccl38a.4*, *saa*, *tnfrsf9a*) were down-regulated (Table 4).
307 Apolipoproteins are a type of high-density lipoproteins, which are capable of direct
308 binding and neutralizing LPS [32], as well as attenuating LPS-induced inflammation by
309 destabilizing cytokine mRNAs [33]. Moreover, the most up-regulated gene, *thrsp*
310 (*thyroid hormone responsive*, fold change: 3.7), encodes a nuclear transcript factor that
311 is responsive to thyroid hormone signal and involved in lipogenesis [34,35]. Thus, it is
312 likely that upon the LPS treatment, the Thrsp is involved in promoting the generation
313 of apolipoproteins, which serve the main role in detoxifying LPS.

314 In the 24 °C group, the LPS challenge up-regulated cytokine expression, which led to
315 both pro-inflammatory (*ly86*, *tlr5b*, *cxcl8a*, *cxcl11.1*, *cxcl18a.1*, *il6*, *il13ra2*, *il34*, *tnfb*,
316 *cebpb*) and anti-inflammatory responses (*il10ra*, *nfkbiaa*, *nfkbiab*, *socs3a*, *socs3b*,
317 *tsc22d3*, *acod1*). The stimulated inflammatory pathways might contribute to activate
318 granulocyte/macrophage differentiation (*cebpa*, *csf1b*, *csf3a*, *csf2rb*, *mydgf*) and
319 neutrophil activities (*ncf4*, *cyba*, *mpx*). Besides, the significantly induced phagosome
320 and vesicular transport transcripts (*marco*, *rhoca*, *rab8b*, *rab20*, *rab32a*, *snx10a*), and
321 lysosomal transcripts (*atp6v1g1*, *atp6v0d1*, *atp6v0ca*, *atp6ap1b*, *atp6v1h*, *atp6ap2*)
322 indicate that LPS triggered the phagosome-lysosome system. Notably, one of the most
323 responsive genes (*fkbp5*) encoding a cis-trans prolyl isomerase [36], showed high
324 increase in expression (fold change 7.6) (Tables 3, 4). Fkbp5 is a protein chaperon as
325 well as a member of immunophilin family with multiple functions, such as protein
326 folding and trafficking, and immunosuppression [37,38]. The high induction of the
327 Fkbp5 gene suggests that it has been involved in mitigating LPS toxicity. A number of
328 Hsp genes (*hspa4l*, *dnajc3a*, *dnajc5ab*, *dnajb11*) was significantly up-regulated post LPS
329 treatment in the 24 °C incubation temperature group (Table 4). The gene *hspa4l*
330 belongs to the *hsp70* family, while *dnajc5ab*, *dnajc3a* and *dnajb11* are homologs of
331 *dnaj/hsp40*. Both *hsp70* and *dnaj/hsp40* are stress-inducible genes and have important
332 roles in the immune response [39,40]. Their significant induction indicates that Hsp
333 transcripts may have contributed to neutralize LPS toxicity.

334 Contrary to the other incubation temperature groups, a limited response to the LPS
335 treatment was found in the 32 °C incubation temperature group. There was no clear

336 sign of apolipoprotein- or inflammatory gene induction, as observed in the other
337 groups. One possibility to explain the lack of this type of response is that some
338 immune effectors were already expressed at sufficiently high levels before LPS
339 challenge in the spleen of fish from the 32 °C incubation temperature group. For
340 instance, the expression of diverse immunoglobulins and/or activated *apoeb*
341 expression (fold change: 6.1) in naïve zebrafish may contribute to alleviate LPS toxicity
342 at 12 hpi of LPS. Besides, the expression of *hsp90aa1.1*, an important Hsp gene
343 involved in activating splenic macrophages [41], was significantly induced (Table 4),
344 likely contributing to the immune response to LPS challenge.

345

346 *The role of epigenetic modifications in thermal imprinting*

347 It is suggested that the epigenetic modifications are involved in determining
348 phenotypic plasticity of fish by thermal environment during the early development
349 [42]. In the present study, several histone modification genes exhibited significant
350 differences in transcript abundances across the incubation temperature groups,
351 including *lysine (K)-specific demethylase 2Ab* (*kdm2ab*), and *Cbp/p300-interacting*
352 *transactivator with Glu/Asp-rich carboxy-terminal domain 4a* (*cited4a*) in the 24 °C
353 group, and *protein arginine methyltransferase 2* (*prmt2*), *H1 histone family member 0*
354 (*h1f0*) in the 32 °C group, as compared to the reference 28 °C group. The gene *kdm2ab*
355 encodes an epigenetic reader protein that interacts with nucleosome and
356 heterochromatin protein 1 (Hp1) through a CXXC/PHD module and recognizes CpG
357 methylation and histone 3 lysine 9 trimethylation (H3K9me3) modification, having a
358 direct role in chromatin silencing [43]. Cited4 is an inhibitor of hypoxia-inducible factor
359 1α (Hif-1α) by binding to CBP/p300 in a reversible manner [44]. The protein arginine
360 methyltransferase PRMT2 has a weak methyltransferase activity on the histone H4
361 [45], while its optimal substrate is still not determined yet [46]. Histone H1 family is
362 not a modifier for DNA or histone but rather a histone linker. It is able to form higher
363 order of chromatin structures in a dynamic manner [47], and its heterogeneity is
364 positively associated with tumor differentiation [48]. A microRNA (miRNA)-related
365 gene, *argonaute RISC catalytic component 2* (*ago2*), was up-regulated in the 24 °C
366 group compared to the reference 28 °C group. Ago2 is capable of binding mature
367 miRNAs, contributing to form RNA-induced silencing complex (RISC) and repressing the
368 translation of mRNAs [49]. The direct involvement of these epigenetic-related genes in
369 the thermal plasticity of the zebrafish immune transcriptome remains to be
370 ascertained.

371 ## Conclusions

372 We found that both low (24 °C) and high (32 °C) embryonic incubation temperatures
373 affected cytokine-, phagocyte- and lymphocyte-related genes in the spleen of adult
374 zebrafish, compared to control temperature (28 °C). The high embryonic incubation
375 temperature (32 °C) also affected more physiological processes, and induced
376 expression of high diversity immunoglobulins. We also showed for the first time that
377 the molecular response to an immune challenge with LPS greatly depended on the
378 prior embryonic incubation temperature. Zebrafish kept at constant 28 °C primarily
379 relied on diverse apolipoproteins, fish from the low embryonic incubation temperature
380 (24 °C) depended heavily on their inflammatory response and phagosome-lysosome
381 system, while the high embryonic incubation temperature group (32 °C) exhibited a
382 limited response to LPS, with only few induced immune transcripts. This is the first
383 study to reveal the long-lasting and distinct effects of temperature during
384 embryogenesis on the immune transcriptome of an adult teleost, which is especially
385 important in the context of climate change.

386

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393

394 ## Author Contributions

395 J.M.O.F., I.B. and Q.Z. designed the experiment. Q.Z. performed the experiments,
396 conducted the RNA sequencing, and drafted the manuscript. Q.Z., J.M.O.F. and I.B.
397 analyzed the data and revised the manuscript.

398

399 **Competing financial interests:** The authors declare no competing financial interests.

400

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514

515 # Tables

516 **Table 1.** Summary of read statistics of sequencing and mapping. A total of 30 libraries,
517 including five LPS-treated replicates and five control replicates from each of three
518 embryonic incubation temperature groups (24 °C, 28 °C, and 32 °C), were single-end
519 sequenced on a NextSeq 500 (Illumina) platform. Q20 represents accuracy of base call
520 > 99%, Q30 represents accuracy > 99.9%.
521

522

	Minimum	Maximum	Mean	Total
Quality control				
Raw reads	25,524,532	36,107,627	31,023,298	930,698,948
Clean reads	24,490,213	34,797,299	29,743,651	892,309,541
≥ Q20 (%)	97.9	98.3	98.1	
≥ Q30 (%)	96.4	97.1	96.8	
Mapping				
Unique	19,142,914	30,021,216	24,872,937	746,188,123
Multiple	2,466,588	5,477,052	3,955,122	118,653,665
Total (%)	82.0	98.2	96.9	

523 **Table 2.** Top 20 most abundant transcripts. BaseMean = mean value of normalized
524 reads counts that were divided by size factor of each library. S.E. = standard error.
525

526

Ensembl ID	BaseMean	S.E.	Symbol	Description
ENSDARG00000097011	1,037,110	111,027	*hbaa1*	hemoglobin, alpha adult 1
ENSDARG00000089087	1,029,072	114,430	*hbba1*	hemoglobin, beta adult 1
ENSDARG00000079078	913,705	98,302	*si:ch211-5k11.8*	si:ch211-5k11.8
ENSDARG00000097238	611,020	63,702	*hbba1*	hemoglobin, beta adult 1
ENSDARG00000063905	224,731	11,693	*mt-co1*	cytochrome c oxidase I, mitochondrial
ENSDARG00000099970	218,173	18,160	*CR383676.1*	CR383676.1
ENSDARG00000037870	212,672	5,383	*actb2*	actin, beta 2
ENSDARG00000069734	204,052	22,348	*hbba2*	hemoglobin, beta adult 2
ENSDARG00000015551	156,861	12,780	*fth1a*	ferritin, heavy polypeptide 1a
ENSDARG00000011166	138,360	10,330	*cahz*	carbonic anhydrase
ENSDARG00000080337	121,307	14,240	*NC_002333.4*	NC_002333.4
ENSDARG00000038643	119,847	8,883	*alas2*	aminolevulinate, delta-, synthase 2
ENSDARG00000069735	114,378	12,852	*hbaa2*	hemoglobin, alpha adult 2
ENSDARG00000063908	112,328	6,707	*mt-co2*	cytochrome c oxidase II, mitochondrial
ENSDARG00000020850	105,247	2,800	*eef1a1l1*	eukaryotic translation elongation factor 1 alpha 1, like 1
ENSDARG00000063912	104,664	6,512	*mt-co3*	cytochrome c oxidase III, mitochondrial
ENSDARG00000095556	101,271	7,319	*CR318588.4*	CR318588.4
ENSDARG00000077777	98,272	4,180	*tmsb4x*	thymosin, beta 4 x
ENSDARG00000037746	79,976	3,137	*actb1*	actin, beta 1
ENSDARG00000077504	74,240	5,253	*si:ch211-103n10.5*	si:ch211-103n10.5

Table 3. Effect of embryonic thermal experience on the spleen transcriptome in adult zebrafish. Representative differentially expressed key immune genes are shown. Pairwise comparisons were performed for 24 °C versus 28 °C groups, and 32 °C versus 28 °C groups.

Symbol	Description	Adjusted p-value		Fold change	
Embryonic incubation temperature 24 °C vs 28 °C, 32 °C vs 28 °C					
tlr21	toll-like receptor 21	0.036	0.002	2.6	3.0
apoeb	apolipoprotein Eb	0.009	< 0.001	3.5	6.1
igl3v1	immunoglobulin light 3 variable 1	0.020	0.045	3.2	2.7
cxcl8a	chemokine (C-X-C motif) ligand 8a	< 0.001	< 0.001	-3.6	-4.2
cxcl11.1	chemokine (C-X-C motif) ligand 11, duplicate 1	< 0.001	< 0.001	-5.0	-4.2
ccl20a.3	chemokine (C-C motif) ligand 20a, duplicate 3	0.004	0.001	-2.8	-2.8
il12ba	interleukin 12Ba	0.024	0.008	-3.2	-3.4
tnfa	tumor necrosis factor a	0.002	0.037	-3.3	-2.0
saa	serum amyloid A	0.001	0.014	-4.1	-2.8
socs1a	suppressor of cytokine signaling 1a	0.009	0.044	-2.2	-1.8
serpinb1l2	serpin peptidase inhibitor	< 0.001	< 0.001	-11.9	-10.4
ncf1	neutrophil cytosolic factor 1	0.033	0.013	-2.2	-2.2
crtam	cytotoxic and regulatory T-cell molecule	0.020	0.007	-2.2	-2.2
Embryonic incubation temperature 24 °C vs 28 °C					
fkbp5	FK506 binding protein 5	< 0.001		-7.5	
ccl35.2	chemokine (C-C motif) ligand 35, duplicate 2	0.019		-3.2	
tagapb	T-cell activation RhoGTPase activating protein b	0.004		-2.2	
nfkbiaa	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a	0.002		-2.4	
jund	JunD proto-oncogene, AP-1 transcription factor subunit	0.039		-1.8	
Embryonic incubation temperature 32 °C vs 28 °C					
rag1	recombination activating gene 1	0.017		3.0	
ciita	class II, major histocompatibility complex, transactivator	0.001		2.6	
cd74a	CD74 molecule, major histocompatibility complex, class II invariant chain a	< 0.001		3.8	
ighv1-4	immunoglobulin heavy variable 1-4	< 0.001		3.4	
ighd	immunoglobulin heavy constant delta	0.009		2.8	
igl3v5	immunoglobulin light 3 variable 5	0.001		3.3	
igl1c3	immunoglobulin light 1 constant 3	< 0.001		3.2	
igl4v9	immunoglobulin light 4 variable 9	0.033		2.8	
igsf9a	immunoglobulin superfamily, member 9a	0.034		2.8	
igic1s1	immunoglobulin light iota constant 1, s1	0.003		2.8	
c1qa	complement component 1, q subcomponent, A chain	< 0.001		2.6	
c1qb	complement component 1, q subcomponent, B chain	0.002		2.3	
c1qc	complement component 1, q subcomponent, C chain	0.001		2.2	
tlr1	toll-like receptor 1	0.026		1.9	
tlr20.2	toll-like receptor 20, tandem duplicate 2	0.010		2.7	
cebp1	CCAAT/enhancer binding protein (C/EBP) 1	0.008		-2.7	
ccl35.1	chemokine (C-C motif) ligand 35, duplicate 1	0.032		-2.2	
il1b	interleukin 1, beta	0.031		-2.4	
il11b	interleukin 11b	0.006		-3.0	
il16	interleukin 16	0.034		-1.9	
atp6v0b	ATPase, H+ transporting, lysosomal V0 subunit b	0.013		-1.8	
atp6v1h	ATPase, H+ transporting, lysosomal V1 subunit H	0.008		-1.8	
psmc3	proteasome 26S subunit, ATPase 3	0.015		-1.7	
psmc4	proteasome 26S subunit, ATPase 4	0.035		-1.6	

533 **Table 4.** Representative list of differentially expressed key immune genes in response
534 to LPS treatment. Spleen transcriptomes at 12 h post LPS injection were compared to
535 their counterparts in controls zebrafish originating from the same embryonic
536 incubation temperature group.

537

Symbol	Description	Adjusted p-value	Fold change
Embryonic incubation temperature 24 °C (LPS vs control)			
mpeg1.2	macrophage expressed 1, tandem duplicate 2	< 0.001	1.8
tlr5b	toll-like receptor 5b	< 0.001	1.6
il6	interleukin 6 (interferon, beta 2)	0.049	2.0
il34	interleukin 34	0.001	1.7
cxcl8a	chemokine (C-X-C motif) ligand 8a	0.016	2.1
cxcl11.1	chemokine (C-X-C motif) ligand 11, duplicate 1	0.018	2.3
cxcl18a.1	chemokine (C-X-C motif) ligand 18a, duplicate 1	0.006	2.1
rab8b	RAB8B, member RAS oncogene family	0.011	1.5
snx10a	sorting nexin 10b	0.006	1.9
atg6v0d1	ATPase, H+ transporting, lysosomal V0 subunit d1	0.008	1.6
atp6v1h	ATPase, H+ transporting, lysosomal V1 subunit H	0.030	1.5
csf1b	colony stimulating factor 1b (macrophage)	0.017	2.3
csf3a	colony stimulating factor 3 (granulocyte) a	0.022	2.0
mydgf	myeloid-derived growth factor	0.049	1.5
ncf4	neutrophil cytosolic factor 4	0.022	1.6
fkbp5	FK506 binding protein 5	< 0.001	7.6
hspa4l	heat shock protein 4 like	0.005	2.7
dnajc3a	DnaJ (Hsp40) homolog, subfamily C, member 3a	0.013	1.7
dnajc5ab	DnaJ (Hsp40) homolog, subfamily C, member 5ab	0.001	1.7
nfkbiaa	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a	0.006	1.7
socs3a	suppressor of cytokine signaling 3a	0.041	1.9
socs3b	suppressor of cytokine signaling 3b	0.049	1.8
cdc20	cell divison cycle 20 homolog	0.004	-1.8
cdkn3	cyclin-dependent kinase inhibitor 3	0.026	-1.7
atg9b	autophagy related 9B	0.036	-1.6
Embryonic incubation temperature 28 °C (LPS vs control)			
apoa1b	apolipoprotein A-Ib	0.002	3.2
apoeb	apolipoprotein Eb	0.007	2.7
apoc1	apolipoprotein C-I like	0.011	2.7
apoa2	apolipoprotein A-II	0.013	2.7
apobb.1	apolipoprotein Bb, tandem duplicate 1	0.032	2.5
apoa1a	apolipoprotein A-Ia	0.041	2.4
cxcl8a	chemokine (C-X-C motif) ligand 8a	0.031	-2.3
cxcl11.1	chemokine (C-X-C motif) ligand 11, duplicate 1	0.004	-3.0
ccl38a.4	chemokine (C-C motif) ligand 38, duplicate 4	0.038	-2.4
il12ba	interleukin 12Ba	0.013	-2.7
saa	serum amyloid A	0.004	-2.6
Embryonic incubation temperature 32 °C (LPS vs control)			
hsp90aa1.1	heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 1	0.039	2.3
c1qa	complement component 1, q subcomponent, A chain	0.020	-2.2
c1qb	complement component 1, q subcomponent, B chain	0.030	-2.0
c1qc	complement component 1, q subcomponent, C chain	< 0.001	-2.2
tlr21	toll-like receptor 21	0.033	-2.7
cd74a	CD74 molecule, major histocompatibility complex, class II invariant chain a	0.003	-2.8

538 # Figure legends

539 **Figure 1.** Experimental design. Zebrafish embryos at the cleavage stage were collected
540 at 28 °C and randomly assigned to one of the three temperature groups (24, 28 and
541 32 °C) for embryogenesis. The water temperature in the 24 and 32 °C groups was
542 gradually adjusted to 28 °C when the larvae reached the first-feeding stage, and
543 maintained at this temperature throughout the experiment. At 100 days post-
544 fertilization, seven fish in each temperature group were intraperitoneally injected with
545 LPS and another seven fish were injected with phosphate-buffered saline as control.
546 RNA was extracted from spleen samples collected at 12 h post-injection. RNA-seq
547 libraries were built and sequenced on a NextSeq 500 platform, and used for
548 differential gene expression analysis.

549 **Figure 2.** Effect of embryonic incubation temperature on the spleen transcriptome in
550 adult zebrafish. (A), volcano plots showing pairwise comparisons of 24 °C vs. 28 °C, and
551 32 °C vs. 28 °C incubation temperature groups. Differentially expressed genes (DEGs;
552 adjusted $P < 0.05$ and $|\text{fold change}| > 1.5$) are displayed in blue (down-regulated) and
553 red colors (up-regulated). (B), Overlapping or unique DEGs from the two
554 aforementioned comparisons. (C), Principal component analysis of DEGs across all the
555 replicates. (D), Left-side heatmap: Clustering of all DEGs; right-side heatmap: clustering
556 of immune-related transcripts. DEGs regulated exclusively in 24 °C or 32 °C group are
557 in blue and red font, respectively, and those significantly regulated in both groups are
558 shown in black. Expression values in the heatmap are centered and scaled by DESeq2
559 normalized read count in the row direction.

560 **Figure 3.** Gene ontology enrichment of differentially expressed genes in the spleen of
561 adult zebrafish originating from different embryonic incubation temperature groups
562 (adjusted p-value < 0.05, modified Fisher exact test). (A) up-regulated and (B) down-
563 regulated biological processes.

564 **Figure 4.** Effect of LPS treatment on the spleen transcriptome of adult zebrafish
565 originating from different embryonic incubation temperatures. (A), Volcano plots of
566 pairwise comparisons (LPS group versus control fish) in each incubation temperature
567 group. Differentially expressed genes (DEGs; adjusted $P < 0.05$ and $|\text{fold change}| > 1.5$)
568 are displayed in blue (down-regulated) and red colors (up-regulated). (B), Heatmap of
569 all DEGs (left side) and immune-related transcripts (right side). Expression values in the
570 heatmap are centered and scaled by DESeq2 normalized read count in the row
571 direction.

572 **Figure 5.** Functional analysis of differentially expressed genes (DEGs) in the spleen of
573 adult zebrafish in response to LPS challenge. The effect of embryonic thermal
574 experience: (A) up- and (B) down-regulated DEGs in samples from fish originating from
575 the three different embryonic incubation temperatures (24, 28 and 32 °C). The DEGs
576 that responded similarly in either two or three temperature groups are marked in grey,
577 and they have not been used for the downstream enrichment analyses. Enrichment in
578 biological processes by (C) up- and (D) down-regulated DEGs. Enrichment in KEGG and
579 Reactome pathways by (E) up- and (F) down-regulated DEGs. Adjusted p-value < 0.05,
580 modified Fisher exact test.

581 **Figure 6.** Diagram summarising the effect of embryonic incubation temperature on the
582 responsiveness of immune transcriptome in the spleen of adult zebrafish. The thermal
583 experience during embryogenesis affected immune transcript levels in the spleen of
584 adult fish. Transcripts of cytokines, and genes related to neutrophil- and T-cell activity
585 were down-regulated in both low (24 °C) and high (32 °C) embryonic incubation
586 temperature groups. Besides, in the 32 °C group a large number of immunoglobulin
587 transcripts were increased, while multiple transcripts involved in endosome-
588 lysosome/proteasome systems were down-regulated. Embryonic thermal experience
589 also had long-term effects on the immune transcriptome responsiveness of spleen to
590 LPS challenge in adult zebrafish. Strikingly, the landscape of immune-related
591 transcriptome upon the LPS challenge has distinctly three specific patterns in the three
592 embryonic incubation temperature groups. In the 28 °C group, a variety of
593 apolipoproteins were induced to counteract the LPS threat, while inflammatory
594 response transcripts were suppressed. In the 24 °C group, the immune response was
595 stimulated with abundant up-regulation of transcripts involved in inflammatory
596 response, endosome-lysosome system, heat shock proteins, neutrophil superoxide and
597 T cell activators, and antibacterial factors, while transcripts involved in pro-autophagy
598 were down-regulated. In the 32 °C group, fish only displayed a limited immune
599 transcriptome response to LPS challenge in spleen.

600

601 ## Supplementary file legends

602 **Supplementary file 1.** Reads statistics of all libraries.
603 **Supplementary file 2.** Identification of an outlier library. A) PCA and B) correlations of
604 control replicates from low embryonic incubation temperature group.
605 **Supplementary file 3.** Differentially expressed genes in the spleen transcriptome
606 affected by the embryonic incubation temperature.
607 **Supplementary file 4.** Enriched biological processes and pathways affected by the
608 embryonic incubation temperature.
609 **Supplementary file 5.** Differentially expressed genes in response to LPS challenge.
610 **Supplementary file 6.** Enriched biological processes and pathways in response to LPS
611 challenge.
612
613 Supplementary files 1 - 6: http://jmofernandes.com/qirui.html
614

615 **Figure 1**

616

617

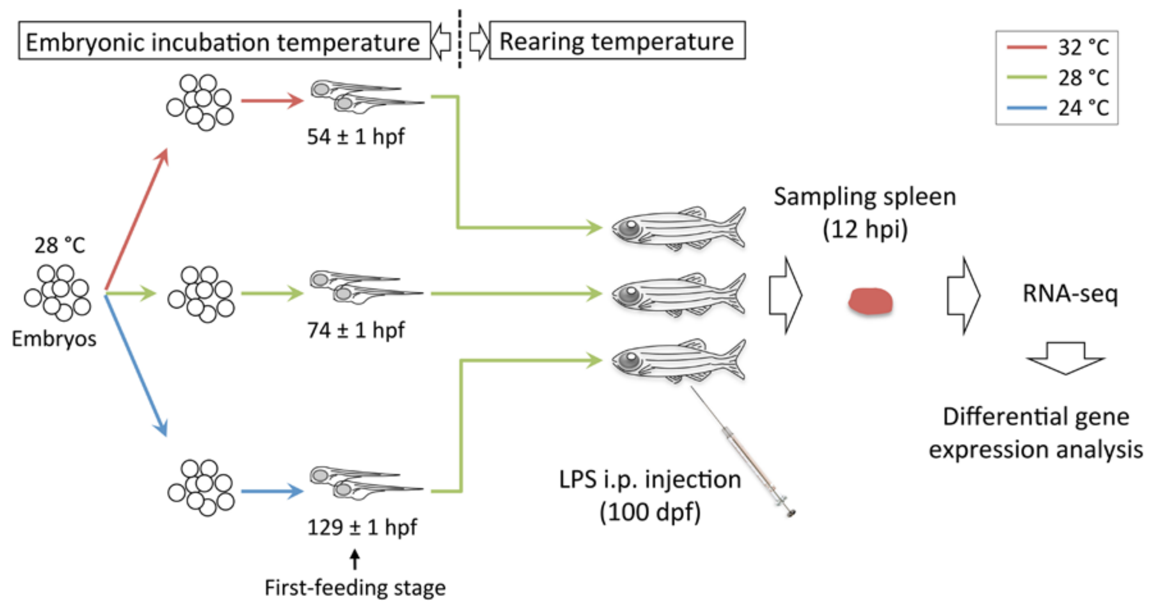

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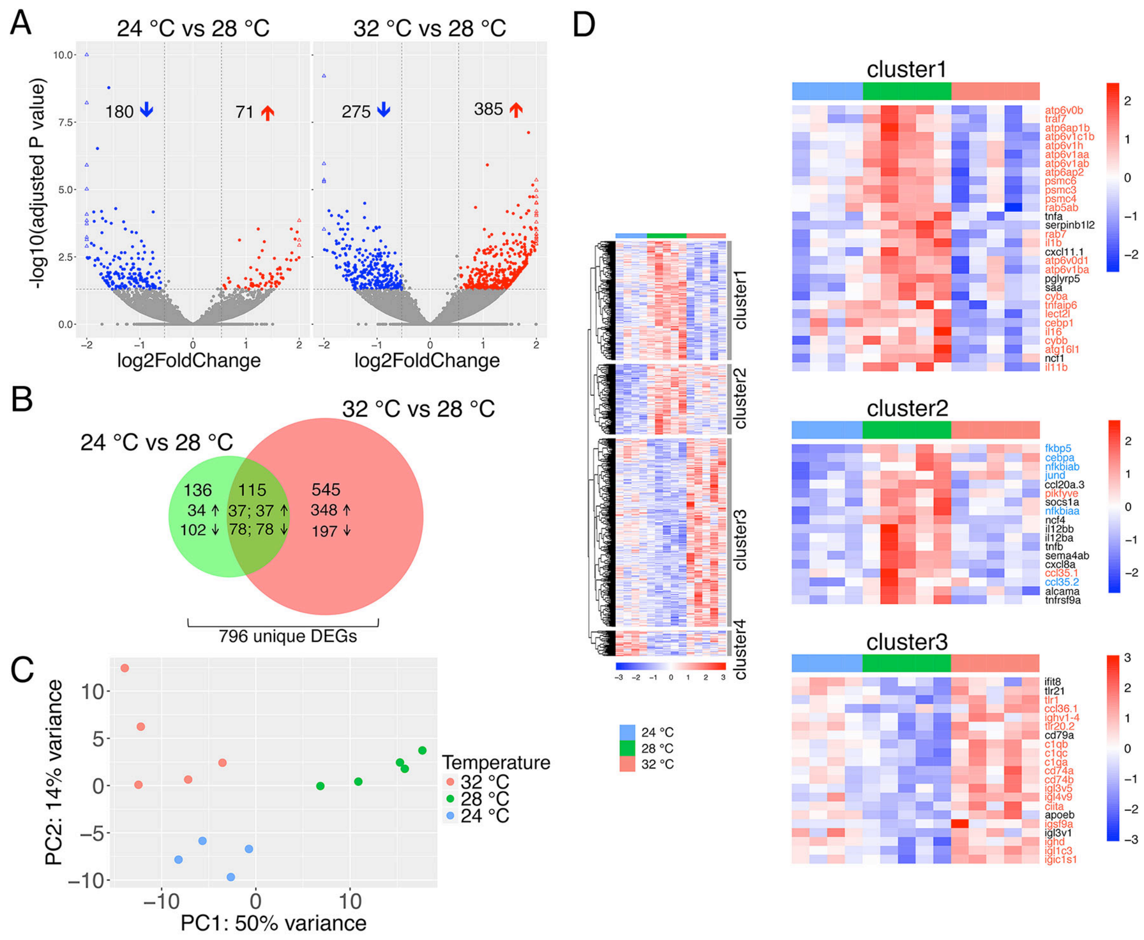
Figure 2



621 **Figure 3**

622

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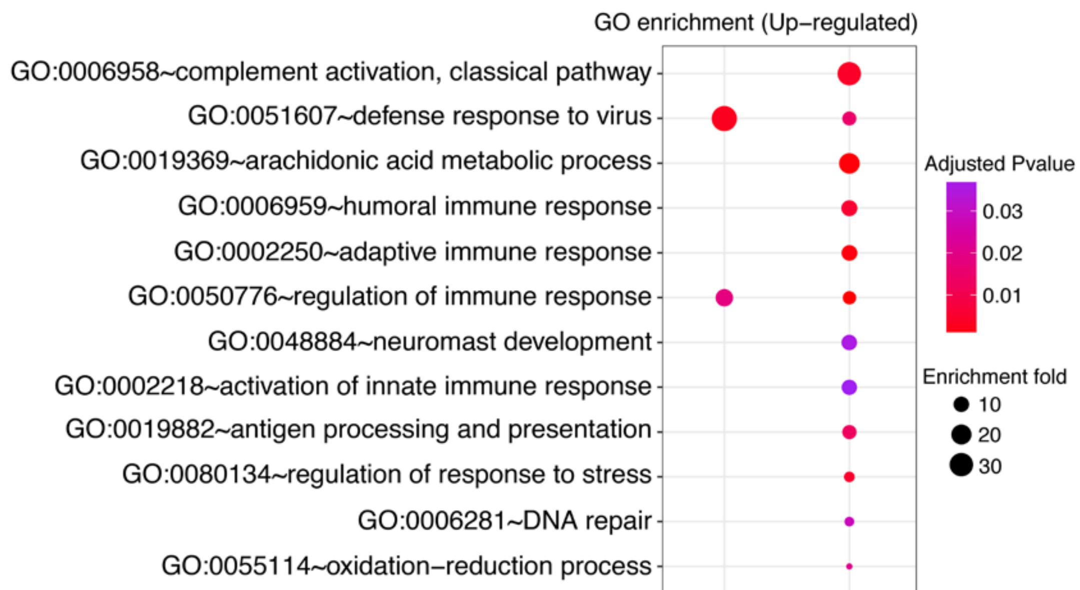

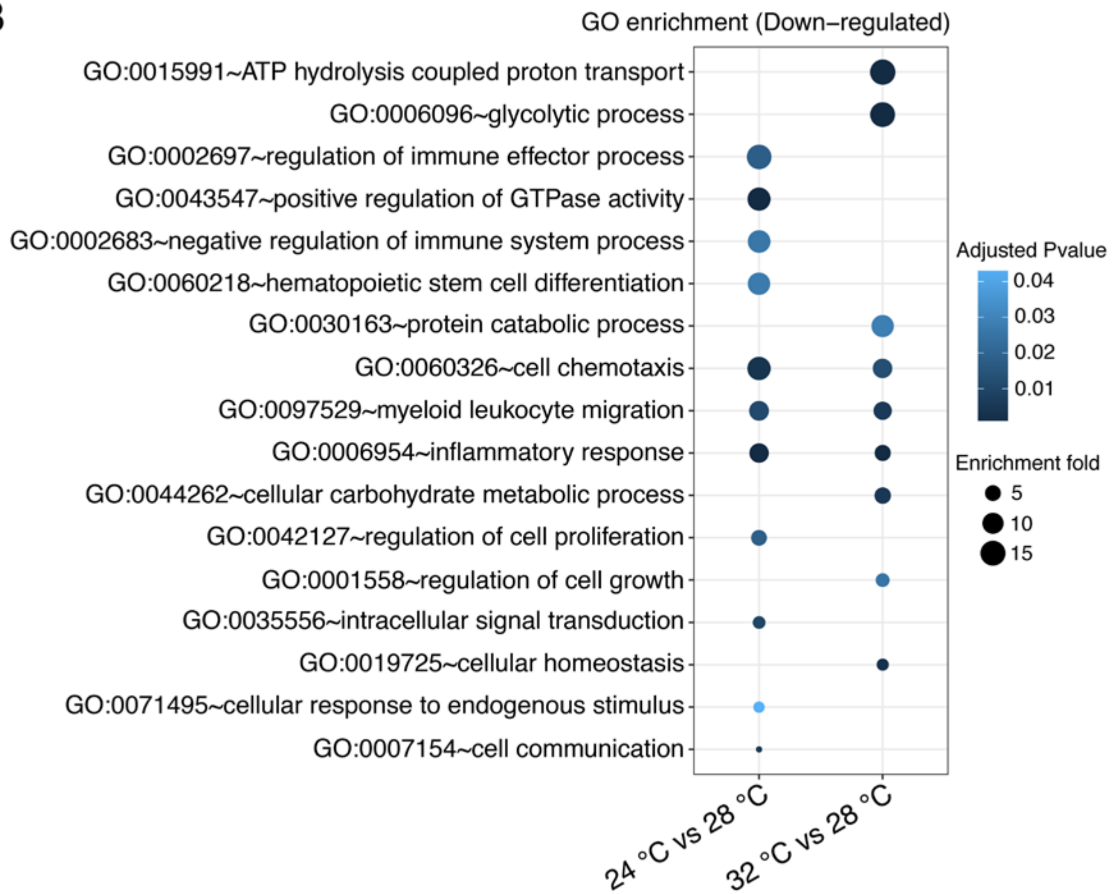

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Figure 4

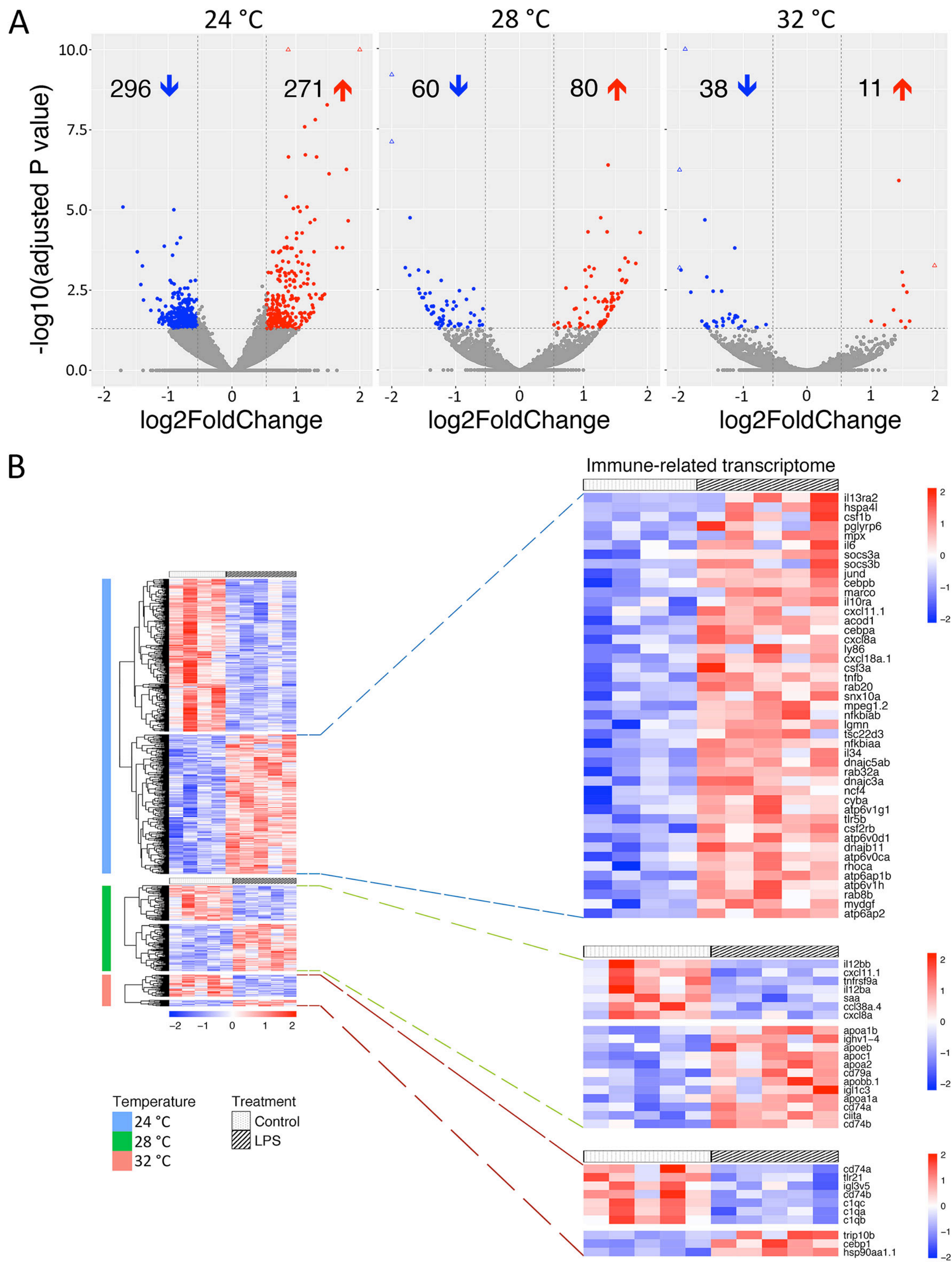

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Figure 5

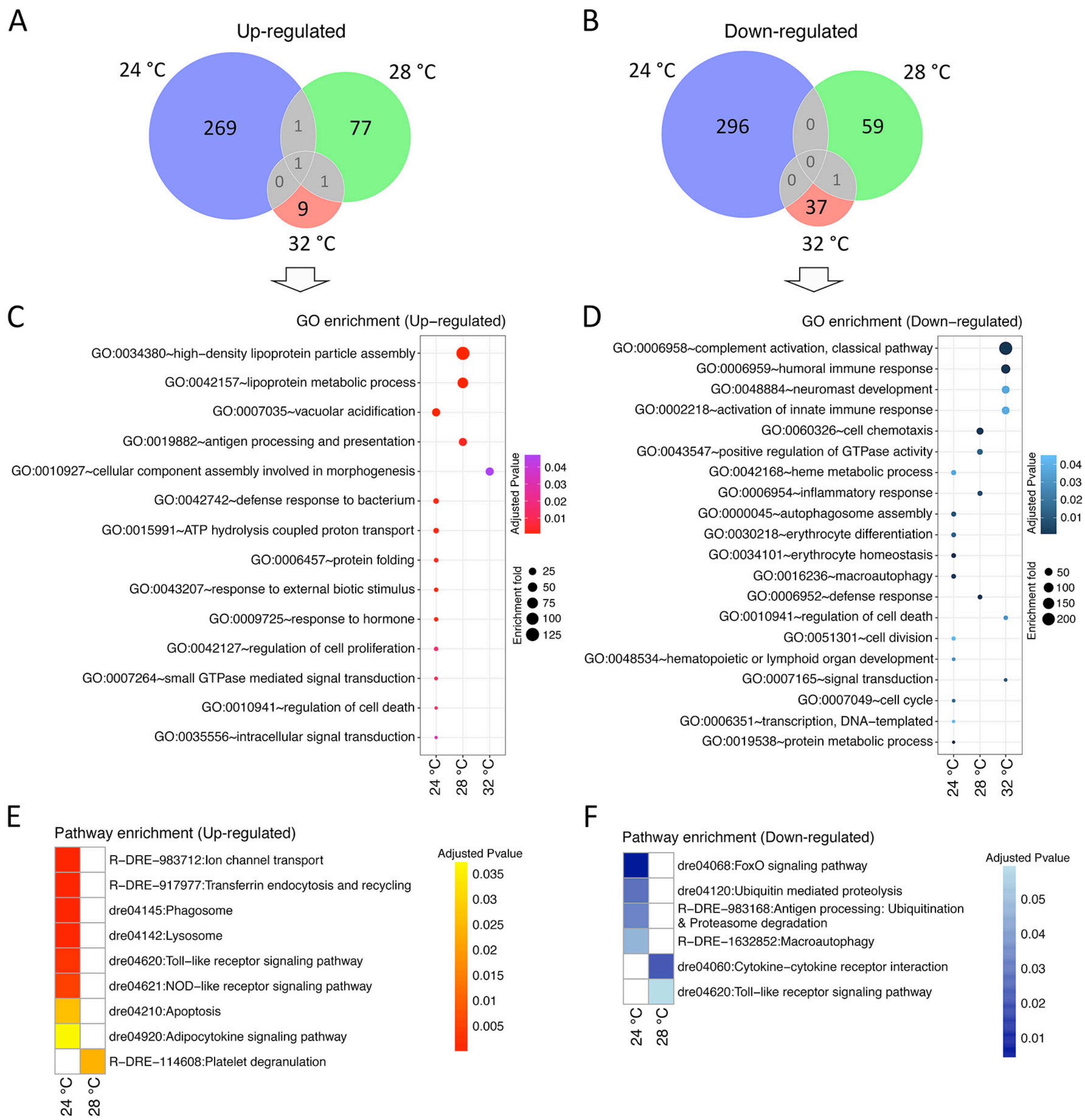

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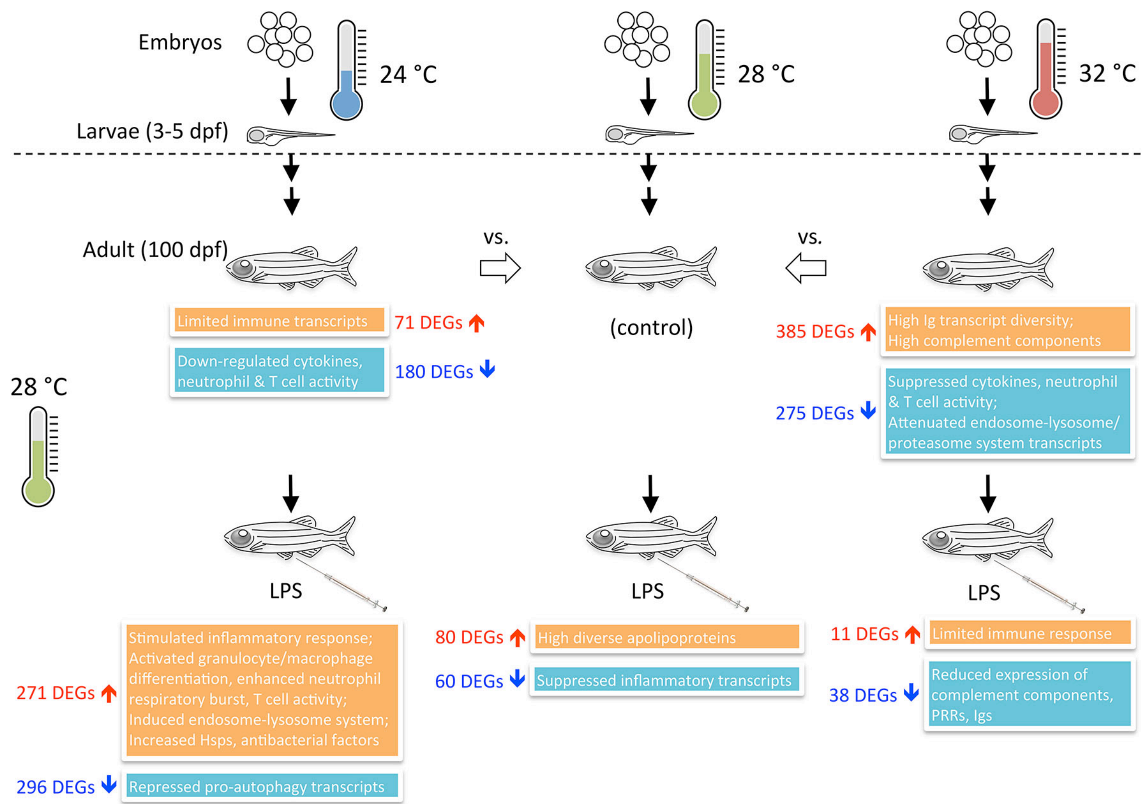
Figure 6



Paper III

Thermal experience during early development modulates microRNA transcriptome in the spleen of adult zebrafish

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Abstract

The thermal plasticity imprinted during early ontogeny has long-term effects on several adult phenotypes but its impact on the immune system is still poorly understood. MicroRNAs (miRNAs) are a class of small non-coding RNAs that are widely involved in regulating the immune response, and their expression can be affected by temperature. In this study, we investigated the effect of developmental thermal environment (24, 28 or 32 °C until 3-5 d post fertilization) on the miRNA transcriptome in spleen of adult fish reared in common garden. Small RNA-seq revealed 150 miRNAs conserved in zebrafish, 130 additional mature miRNAs known in other species, and 99 novel miRNA candidates. Thirty-two miRNAs were differentially expressed in the spleen of fish from the 32 °C embryonic incubation temperature group compared to 28 °C group; of these, 29 were up-regulated and three were down-regulated. The enrichment analysis of the predicted targets revealed that the immune status was significantly affected ("endocytosis", "vesicle-mediated transport", "negative regulation of leukocyte activation" and "induction of positive chemotaxis"), as was the cell cycle ("cell cycle"). No miRNAs were significantly differentially expressed in the spleen of fish from the 24 °C embryonic incubation temperature group compared to the 28 °C group. Lipopolysaccharide challenge regulated transcript levels of three miRNAs only in the spleen of fish kept at constant 28 °C, rather than in fish that experienced low or high temperatures during embryogenesis. Their target genes enriched in immune processes, such as "endocytosis", "vesicle-mediated transport", "cytokine production", "NIK noncanonical NF-κB signaling". In summary, a high embryonic incubation temperature (32 °C) had a long-term effect on miRNA expression in adult zebrafish spleen, while low embryonic incubation temperature had minor effect.

Abbreviations

LPS: lipopolysaccharide; DEG: differentially expressed gene; dpf: days post-fertilization; GO: gene ontology; KEGG: Kyoto encyclopaedia of genes and genomes; PCA: principal component analysis; IL: interleukin; IFN: interferon; TNF: tumor necrosis factor; NF-κB: nuclear factor κ-light-chain-enhancer of activated B cells; miRISC: miRNA-induced silencing complex; Ago: Argonaute; 3’ UTR: 3’ untranslated region; i.p.: intraperitoneally

Key words

developmental plasticity, immunity, lipopolysaccharide, miRNA, teleost

Introduction

The innate and adaptive immune systems of jawed fishes develop early during the ontogeny. In zebrafish, macrophages and neutrophils become mature as early as 1-2 days post fertilization (dpf) [1], and transcripts of the immune marker genes *IKAROS family zinc finger 1* (*ikaros*), *recombination activating gene 1* (*rag-1*), *T-cell receptor alpha constant region* (*tcrac*) and *immunoglobulin light chain constant region* (*iglc*) are detectable from 1-3 dpf onwards [2]. The spleen is an important secondary lymphoid organ that contains abundant mature myeloid cells and lymphocytes, and has a crucial function in the immune response [3]. Spleen size is positively associated with the resistance to *Flavobacterium psychrophilum* challenge in rainbow trout [4] and splenic melanomacrophages are able to phagocytose infectious materials and scavenge blood-borne pathogens [5]. In common carp (*Cyprinus carpio*), hundreds of immune-related genes were significantly regulated in the spleen following *Aeromonas hydrophila* infection [6].

Compared to adults, young fish are particularly susceptible to environmental temperature, and the thermal plasticity during the early ontogeny can be reflected in an adult phenotype [7,8], including metabolic enzyme activity [9], muscle growth [10], swimming performance [11], thermal tolerance [12], or cardiac anatomy [13]. However, little is known about the thermal plasticity of the immune system in fish, with the exception of a recent study in sea bream (*Sparus aurata,* L.). It showed that elevated temperatures during embryonic or larval incubation led to a decreased number of melanomacrophage centers and lower level of dopachrome tautomerase transcripts in the pronephros of adult sea bream in response to acute confinement stress [14]. Recently, we found that a short exposure to high (32 °C for 3 days) or low (24 °C for 5 days) embryonic incubation temperature resulted in different transcriptomic profiles of homeostatic and stimulated immune processes in the spleen of adult zebrafish (100 dpf) compared to the control temperature (28 °C) [15]. Nonetheless, the molecular basis of the long-term effect of early embryonic temperature on immune homeostasis and response in adult fish is hitherto poorly understood.

77 MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs (ncRNAs) of
78 approximately ~22 nucleotides (nt) in length, which negatively regulate mRNAs at the
79 post-transcriptional level [16]. miRNAs play important roles in regulating development
80 and differentiation of immune cells, antibody production and inflammatory cytokine
81 release [17,18]. For instance, miR-150, miR-223, and the miR-17-92 cluster are
82 involved in the normal development and differentiation of lymphoid and myeloid cells
83 [19-21], and miR-155 and miR-181a are implicated in antibody production and T cell
84 receptor signaling, respectively [22,23]. Some miRNAs, such as miR-148, miR-214, miR-
85 3570 and miR-8159 are negative regulators of the MyD88-mediated nuclear factor κ-
86 light-chain-enhancer of activated B cells (NF-κB) signaling pathway to avoid excessive
87 inflammation after pathogen infection [24]. Previous studies have demonstrated that
88 miRNAs modulate transcript plasticity under different developmental temperatures in
89 fish [25], and have long-term effects from early ontogeny to adulthood [26,27].
90 However, it is still unknown whether miRNAs are involved in the long-term effects of
91 developmental temperature on the immune system of adult fish.

92 In the present study, we characterized miRNA transcriptome and its predicted
93 targetome in spleen of adult zebrafish, originating from three different embryonic
94 incubation temperatures, and challenged with lipopolysaccharide (LPS) in their
95 adulthood.

96

97 # Materials and methods

98 ## *Ethics statement*

99 All animal procedures were conducted in compliance with the guidelines provided by
100 the Norwegian Animal Research Authority (FOTS ID 13900) and approved by the Nord
101 University (Norway) ethics committee.

102

103 ## *Experimental design*

104 Zebrafish embryos were collected at 28 °C and incubated at either of 24 °C, 28 °C or
105 32 °C during embryogenesis (129 ± 1, 74 ± 1 and 54 ± 1 h at 24, 26 and 28 °C,
106 respectively), as previously reported [28]. From first feeding onwards, all fish were
107 maintained at 28 °C. At 100 dpf, 7 young female adults from each group were
108 intraperitoneally (i.p.) injected with 2 µl of 50 mg/ml LPS *Pseudomonas aeruginosa* 10
109 (Sigma-Aldrich, USA), and another 7 females were i.p. injected with phosphate-
110 buffered saline (Sigma-Aldrich, USA) as control. At 12 h post-injection (hpi) the spleen
111 was sampled. Small RNAs of spleen from each temperature group, with and without
112 LPS challenge, were sequenced and their miRNA transcriptomic profiles were
113 compared.

114

115 ## *Zebrafish maintenance, sampling and total RNA isolation*

116 Zebrafish (AB strain) were maintained in a recirculating aquatic system with
117 temperature of 28 ± 0.5 °C, photoperiod of 12 h light: 12 h dark. Embryos and larvae
118 were kept in Petri dishes, and 14 dpf juveniles were transferred to 3 L nursery tank
119 (Pentair, USA) until 30 dpf and then to a recirculating system with a slow water flow
120 (Aquatic Habitats, USA). Fish were fed SDS (Special Diets Services, SDS, UK). The
121 spleens were dissected immediately following fish euthanasia, snap-frozen in liquid
122 nitrogen, and further stored at -80 °C. Total RNA was extracted using PicoPure RNA
123 isolation kit (ThermoFisher Scientific, USA) with some changes. The spleen was
124 immersed in 100 µl extraction buffer and the cells were dissociated using a 1 ml
125 pipette tip. The cell homogenate was incubated at 42 °C for 30 min, and then
126 centrifuged at 3,000 × g for 2 min. The supernatant was transferred to a new
127 microcentrifuge tube and 100 µl of 70% ethanol were added before transfer to the

128 preconditioned purification column and centrifugation at 100 × g for 2 min. The RNA
129 was sequentially washed by wash buffers 1 and 2, and eluted in 30 µl elution buffer.
130 RNA quality and concentration were assessed using the High Sensitivity RNA
131 ScreenTapes on a TapeStation 2200 (Agilent Technologies, USA).

132

133 *Library preparation and RNA sequencing*

134 From each sample, 50 ng of total RNA was taken and the libraries were prepared using
135 the NEXTflex Small RNA-Seq Kit v3 (Bioo Scientific, USA) following the manufacturer's
136 protocol. RNAs were sequentially ligated with 3' 4N and 5' 4N adenylated adapters,
137 and reverse transcribed into first-strand cDNA. The cDNA was amplified with universal
138 and barcoded primers for 22 cycles on a thermocycler (ABI, USA) using the following
139 cycling parameters: 95 °C for 2 min; 95 °C for 20 s, 60 °C for 30 s, 72 °C for 15 s, 22
140 cycles; 72 °C for 2 min. The size of ligated miRNAs (~150 bp) was selected by
141 electrophoresis using a 10% TBE-PAGE gel. After size selection, the quality and
142 concentration of small RNA libraries were determined using the High Sensitivity RNA
143 ScreenTapes on a TapeStation 2200 (Agilent Technologies, USA). Thirty libraries were
144 sequenced on a single high-output flow cell (single-end, 75 bp) using the NextSeq 500
145 platform (Illumina, USA), at Oslo University Hospital (Norway).

146

147 *Bioinformatic analyses and statistics*

148 The scheme of the procedure is given in Fig. 1. The quality of raw reads was examined
149 using FastQC [29], followed by adapter trimming using cutadapt [30] and removal of
150 low quality reads by FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Bowtie
151 [31] was used to first align the clean reads to the mature miRNAs of zebrafish, and
152 then to align them to the known miRNA sequences from another 15 teleosts
153 (*Astatotilapia burtoni*, *Cyprinus carpio*, *Electrophorus electricus*, *Fugu rubripes*, *Gadus*
154 *morhua*, *Hippoglossus hippoglossus*, *Ictalurus punctatus*, *Metrizclima zebra*,
155 *Neolamprologus brichardi*, *Oryzias latipes*, *Oreochromis niloticus*, *Pundamilia nyererei*,
156 *Paralichthys olivaceus*, *Salmo salar*, and *Tetraodon nigroviridis*), and three model
157 species (*Drosophila melanogaster*, *Mus musculus*, *Homo sapiens*) from miRBase 22
158 release (http://www.mirbase.org) using the following parameters: "-n 1 -e 80 -l 18 -k 1
159 -m 10000 --best --strata". Unaligned reads were mapped against other zebrafish

160 ncRNAs that were downloaded from Ensembl release 93 [32] and Rfam 14.0 [33], and
161 the aligned reads were discarded. The remaining unaligned reads from each library
162 were first pooled by group, used to identify novel miRNAs using miRDeep2 [34], and
163 then mapped to novel miRNAs for quantification. The miRDeep2 results with
164 miRDeep2 score ≥ 5, total read count ≥ 200 and randfold p-value < 0.05 were
165 designated as novel miRNA candidates, and redundant novel miRNAs between groups
166 were collapsed. The aligned reads were counted using featureCounts [35]. All
167 identified known miRNAs from zebrafish plus 18 species and novel miRNAs were
168 combined and filtered using the following two criteria: i) minimum threshold of 100
169 reads in all replicates in each temperature group comparison (5 treatment and 5
170 controls replicates), and ii) presence in at least three out of five replicates of a given
171 temperature/challenge group (either treatment or control, the one that having higher
172 mean value of DESeq2 normalized reads). The retained miRNAs and read counts were
173 used as an input to DESeq2 for differential expression analysis [36]. miRNAs were
174 considered to be differentially expressed with a significance threshold of adjusted p-
175 value < 0.05 (Benjamin-Hochberg method) and a |fold change| ≥ 2. The 3’-UTRs of
176 zebrafish genes were downloaded from Ensembl 93/GRCz11 [32] and used to predict
177 targets of DE miRNAs using miRanda with the parameters: minimum matching score
178 140, maximum energy score -20, strict 5’ seed pairing [37]. Target genes were further
179 subjected to Gene Ontology (GO) biological process, Kyoto Encyclopedia of Genes and
180 Genomes (KEGG) pathway, and Reactome pathway enrichment analyses using DAVID
181 [38], with the significance threshold of modified Fisher exact p-value < 0.05.
182 Correlations and significance between DE miRNAs and differentially expressed genes
183 (DEGs) from our previous unpublished RNA-seq data [15] were calculated using R
184 functions cor and cor.test, respectively, with a p-value <0.05, and networks were built
185 in Cytoscape [39].

186

187 # Results

188 ## *Overview of the spleen miRNA transcriptome in zebrafish*

189 A total of 457,053,296 raw reads were obtained and 97.2% had a quality ≥ Q30. After
190 trimming adapters and removing low-quality reads, 345,070,413 clean reads retained
191 (Table 1). There were three peaks at 22 nt, 29 nt, and 34 nt (Fig. 2A). A total of
192 34,748,205 and 3,285,981 reads aligned to mature miRNAs from zebrafish and other
193 18 species in miRBase, respectively, composing 11.0% of the total clean reads
194 (Supplementary file 1). Other ncRNAs (rRNA, tRNA, snRNA, snoRNA, and others)
195 accounted for 31.7% of clean reads (Fig. 2B).

196 Fifty-three novel miRNAs, mapping to 78 genomic locations, were identified with high
197 confidence based on the miRDeep2 score (Table 2, Supplementary file 2). A total of
198 1,036,344 reads (0.3% of all clean reads) accounted to these novel miRNAs.

199 Fourteen miRNAs composed the top-ten abundant miRNAs in all the six experimental
200 groups (Fig. 3). dre-let-7a-5-5p, dre-miR-92a-3p and dre-miR-21 were the three most
201 abundant miRNAs in each group. Nonetheless, some miRNAs showed different
202 abundance between embryonic incubation temperatures, as well as between LPS
203 treatment and control. For instance, dre-let-7a-5-5p and dre-miR-92a-3p were
204 differentially expressed between control replicates of three embryonic incubation
205 temperature groups, while dre-miR-92a-3p was much more abundant after LPS
206 treatment in 32 °C group (Fig. 3). Raw data were deposited at Gene Expression
207 Omnibus (accession GSE121164).

208

209 ## *Differentially expressed miRNAs and their target genes*

210 Thirty miRNAs were differentially expressed (DE) between the 32 °C and 28 °C
211 embryonic incubation temperature groups (27 up- and 3 down-regulated). It included
212 24 known zebrafish miRNAs (23 up-/1 down-regulated), 3 novel zebrafish miRNAs
213 (2 up-/1 down-regulated), and 3 miRNAs known in other species (2 up-/1 down-
214 regulated). dre-miR-733-5p transcript abundances showed the highest difference
215 between the groups (344-fold up-regulation in the 32 °C group; Fig. 4A). No DE miRNAs

216 were found in the 24 °C versus 28 °C embryonic incubation temperature group
217 comparison (Supplementary file 3).

218 The effect of LPS treatment on the spleen miRNA transcriptome was moderate or
219 negligible in the three incubation temperature groups (Supplementary file 3). Three DE
220 miRNAs (2 up-/1 down-regulated) were identified in the 28 °C group, including one
221 zebrafish miRNA (dre-miR-25-5p, up-regulated) and two miRNAs known in other
222 species (ssa-miR-30d-2-3p up- and hsa-miR-3150b-5p down-regulated; Fig. 4B). No DE
223 miRNAs were found in other temperature groups.

224 A total of 8,561 potential targets (4,313 unique) of all the 33 DE miRNAs were
225 predicted (Table 3, Supplementary file 4). In the embryonic incubation temperature
226 comparison (32 °C versus 28 °C groups), 7,669 potential target genes (4,278 unique)
227 were identified. There was a significant over-representation in 15 GO biological
228 processes in the targets of down-regulated DE miRNAs, and 4 GO biological processes
229 were over-represented in the targets of up-regulated DE miRNAs (Fig. 5A,
230 Supplementary file 5). Predicted targets of the down- and up-regulated DE miRNAs
231 were significantly enriched in 12 and 3 pathways, respectively. For the both groups of
232 DE miRNAs, their predicted targets were enriched in the same pathway
233 "Phosphatidylinositol signaling system" (Fig. 5B, Supplementary file 5).

234 The three DE miRNAs in the 28 °C LPS-treated versus 28 °C control groups comparison
235 zebrafish had 892 putative targets (622 unique). The targets of the down-regulated
236 miRNA, hsa-miR-3150b-5p homolog, were enriched in 9 GO biological processes and 4
237 pathways, while the targets of the up-regulated DE miRNAs were enriched in "protein
238 phosphorylation" biological process and no pathways enrichment was significant
239 (Fig. 5, Supplementary file 5).

240

241 *Integrative analysis of expressed miRNAs and their potential targets*

242 Predicted target genes of DE miRNAs were compared with DEGs obtained in the
243 previous study [15]. In the 32 °C vs 28 °C incubation temperature groups comparison,
244 74 DEGs (12 up-/62 down-regulated) were among the predicted targets (Fig. 6A). A
245 single GO biological process ("signal transduction"), and 11 GO terms and one KEGG

246 pathway were over-represented in the up-regulated and down-regulated DEGs,
247 respectively, predicted as targets of DE miRNAs (Table 4). In the 28 °C LPS vs control
248 comparison, three DEGs (all down-regulated) were among the predicted targets
249 (Fig. 6B) and no significant enrichment was found in GO terms or pathways.

250 The potential regulatory relationships between DE miRNAs and their respective DE
251 targets are shown in Fig. 7. In the comparison Control: 32 °C vs 28 °C, 27 miRNA/mRNA
252 pairs between up-regulated miRNAs and down-regulated DEGs showed a significant
253 negative correlation ($p < 0.05$). Another four miRNA/mRNA pairs between down-
254 regulated miRNAs and up-regulated DEGs showed significantly negative correlations
255 (Fig. 7, Supplementary file 6). No significant miRNA/mRNA correlations were observed
256 in the comparison 28 °C: LPS vs control.

257

258 ## Discussion

259 ### *Embryonic temperature has a long-term effect on the spleen miRNA*
260 ### *transcriptome*

261 Elevated temperature (32 °C) during the short period of embryonic incubation showed
262 long-term consequences in the spleen miRNA transcriptome of adult zebrafish,
263 resulting in altered expression of 30 DE miRNAs, as compared to the control group
264 incubated at 28 °C during the embryogenesis. Two of these DE miRNAs, miR-130b and
265 miR-451, showed different expression profiles in larvae and liver of juvenile Atlantic
266 cod upon different thermal experiences during the embryonic incubation [26].
267 Enrichment analysis of predicted target genes of 30 DE miRNAs suggested that the
268 immune function and cell proliferation could be affected by the observed differences
269 in the miRNA transcriptome between temperature groups. For instance, miR-217 is a
270 positive modulator of the germinal center B cells that increases the generation of
271 class-switched antibodies and the frequency of somatic hypermutation [40].
272 Differentially expressed miRNAs such as miR-10, miR-200a and miR-18a are regulators
273 determining the differentiation of naïve T cells either into Th17 or into regulatory T
274 cells [41-43]. let-7, miR-24 and miR-194 negatively regulate NF-κB signaling [44-46],
275 while miR-125a and miR-130b are positive regulators [47,48]. miR-130b, miR-194 and
276 miR-203 inhibit cell proliferation and/or induce cell cycle arrest even cell apoptosis [49-
277 51], while miR-27a promotes the proliferation and inhibits apoptosis [52]. Moreover,
278 miR-24, miR-125a and miR-181c are critical modulators in hematopoiesis [53-55], and
279 their up-regulation suggests that hematopoietic stem cells could be affected during
280 embryonic incubation under 32 °C.

281 None of DE miRNAs were identified in fish from 24 °C embryonic incubation
282 temperature compared to those kept at constant 28 °C. One possibility is that even if
283 low temperature had an effect in larval zebrafish [28], it may have declined later in
284 ontogeny. An alternative is that zebrafish is much more sensitive to high (32 °C)
285 temperature rather than to low temperature (24 °C). This can be partially supported
286 from another study that even acclimation of zebrafish to 10 °C for 10 days only had a
287 minor effect on miRNA expression in the brain [56].

288

289 *miRNAs and thermal plasticity of the immune response to LPS challenge*

290 miRNAs have important roles in regulating immune response to bacteria in teleost fish
291 [57]. Several immune processes were enriched from the target genes of 3 DE miRNAs
292 from LPS-challenged fish kept at constant 28 °C (Fig. 5), indicating that a response was
293 initiated and miRNAs may have been involved. For instance, miR-30d is a negative
294 regulator of cell proliferation in tumorigenesis, it targets the 3'-UTR of tumor
295 suppressor p53, affecting numerous downstream genes which are involved in the
296 regulation of cell cycle and cell death [58]. While another miRNA, miR-25, promotes
297 cell proliferation and protects cells against TNF-related apoptosis-inducing ligand-
298 induced apoptosis [59]. However, none of miRNAs changed expression significantly in
299 response to LPS challenge in the spleen of zebrafish from embryonic temperature of 24
300 °C or 32 °C. Nonetheless, miR-542-3p was up-regulated with LPS treatment, although
301 not significantly. It is involved in negatively regulating COX-2 expression and
302 prostaglandin synthesis [60], a key pro-inflammatory cytokine, and inhibits cell
303 proliferation through inducing G1 and G2/M cell cycle arrest [61].

304

305 ## Conclusions

306 We found that a high embryonic incubation temperature (32 °C) had a long-term effect
307 on the spleen miRNA expression profiles of adult fish compared to the reference
308 embryonic incubation temperature (28 °C). Putative targets of these DE miRNAs are
309 known to be involved various immune activities including endocytosis, inflammatory
310 response, lymphocyte differentiation, and cell apoptosis. The low embryonic
311 incubation temperature (24 °C) had a minor effect on the miRNA transcriptome of
312 adult fish in both LPS-challenged and control individuals. Only three miRNAs
313 responded to LPS challenge in the spleen of adult fish kept at constant reference
314 temperature (28 °C) and their target genes are likely involved in the immune response.
315 Taken together, our data indicate that miRNAs may have a role in regulating the
316 thermal plasticity of immune gene expression in zebrafish spleen.
317

318
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321 (Nord University, Norway) for his assistance with gel-based miRNA size selection. This
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324

325
Author Contributions

326 J.M.O.F., I.B. and Q.Z. designed the experiment. Q.Z. performed the experiment,
327 conducted the RNA sequencing, and drafted the manuscript. Q.Z., J.M.O.F. and I.B.
328 analyzed the data and revised the manuscript.

329

330 **Competing financial interests:** The authors declare no competing financial interests.
331

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473

474 # Tables

475 **Table 1.** miRNA sequencing and mapping statistics. A total of 30 libraries were
476 sequenced, including 5 LPS-treatment replicates and 5 controls from each temperature
477 group. Q30 represents a base call accuracy > 99.9%. Clean reads were adapter-
478 trimmed and low-quality reads were removed.
479

480

	Mean	Total
Quality control		
Raw reads	15,235,110	457,053,296
≥ Q30 (%)	97.2	97.2
Clean reads	11,502,347	345,070,413
Mapping		
Zebrafish	1,158,274	34,748,205
Other 18 species	109,533	3,285,981
Novel miRNA	34,545	1,036,344
Total (%)	11.3	11.3

481 **Table 2.** Top 5 novel mature miRNAs with highest estimated true positive rate
482 predicted in each group. miRDeep2 score > 5.0, total read count ≥ 200, randfold p-
483 value < 0.05. Estimated true positive is the estimated probability that the novel miRNA
484 with a particularly miRDeep2 score higher is a true positive. Read count is the number
485 of reads for a predicted mature miRNA. Consensus mature miRNA sequences are
486 inferred from deep sequencing reads.
487

488

Novel miRNA id	miRDeep2 score	Estimated true positive	Length	Read count	Consensus mature sequence
24 °C control					
chr3_31903	346.4	49 +/- 16%	22	468	ugccucaguccaaauacacccu
chr3_34157	5.1	68 +/- 9%	25	211	aucaguggaggcggaugauugguuu
chr3_34620	1700.1	49 +/- 16%	23	3280	ucaucccgaaagcaccucccucc
chr4_36463	5239.9	49 +/- 16%	23	10224	cguggcgcgacaggggguggacug
chr9_46584	8938.6	49 +/- 16%	22	16593	ccgccccgucucugcuaccuca
24 °C LPS					
chr1_313-4	863	45 +/- 18%	23	1515	ggauagaaucagcggagcgggga
chr1_313-5	826.8	45 +/- 18%	23	1515	ggauagaaucagcggagcgggga
chr2_19816	5.4	64 +/- 11%	23	228	agacaggugguguuugguuagggu
chr3_31903	385.1	45 +/- 18%	22	558	ugccucaguccaaauacacccu
chr3_34620	3244.9	45 +/- 18%	22	6338	ucaucccgaaagcaccucccuc
28 °C control					
chr1_313-1	115.1	80 +/- 6%	23	222	ggauagaaucagcggagcgggga
chr1_313-9	172	80 +/- 6%	23	222	ggauagaaucagcggagcgggga
chr1_313-2	115.1	80 +/- 6%	23	222	ggauagaaucagcggagcgggga
chr1_313-3	115.5	80 +/- 6%	23	222	ggauagaaucagcggagcgggga
chr1_313-6	115.1	80 +/- 6%	23	222	ggauagaaucagcggagcgggga
28 °C LPS					
chr1_173	6.1	55 +/- 12%	21	287	ucagcacucggacagccucuu
chr1_395	5760.3	52 +/- 13%	23	11155	cuccacugcucugccuccccuca
chr1_775	454.4	52 +/- 13%	23	384	ugacuuuucucuggucugcacag
chr4_35497-1	1470.1	52 +/- 13%	22	2768	ggauagaaucagcggagcgggg
chr4_35497-2	1414.4	52 +/- 13%	22	2768	ggauagaaucagcggagcgggg
32 °C control					
chr1_443	1923.3	45 +/- 10%	22	3673	cuccacugcucugccuccccuc
chr3_33494	121.6	45 +/- 10%	22	190	aaucgguacccuugacuuucau
chr3_34620	1460.4	45 +/- 10%	23	2858	ucaucccgaaagcaccucccucc
chr4_35497-1	1404.3	45 +/- 10%	22	2606	ggauagaaucagcggagcgggg
chr5_36671	552.9	45 +/- 10%	22	199	augacucaaacccgagggcucg
32 °C LPS					
chr1_313-8	1164.4	40 +/- 12%	23	2173	ggauagaaucagcggagcgggga
chr1_313-3	1139.7	40 +/- 12%	23	2173	ggauagaaucagcggagcgggga
chr1_313-7	1147.5	40 +/- 12%	23	2173	ggauagaaucagcggagcgggga
chr1_849	2730.9	40 +/- 12%	21	5179	cuccacugcucugccuccccu
chr3_34620	2319.6	40 +/- 12%	23	4513	ucaucccgaaagcaccucccucc

489 **Table 3.** Differentially expressed miRNAs and the number of their potential targets.
490 BaseMean: mean value of normalized read counts that were divided by library size
491 factor.
492

493

miRNA	BaseMean	Adjusted p-value	Fold change	Target number
Control 32 °C vs control 28 °C: up-regulated				
dre-miR-7a	88	< 0.001	109	190
dre-miR-10a-5p	48	< 0.001	148	159
dre-miR-24-3-5p	77	0.003	160	147
dre-miR-25-5p	27	0.003	113	519
dre-miR-27a-3p	20	0.010	58	407
dre-miR-100-2-3p	21	0.020	56	56
dre-miR-122-3p	4255	0.003	10	466
dre-miR-181c-5p	39	< 0.001	99	640
dre-miR-203b-3p	131	0.008	78	160
dre-miR-217-5p	22	0.007	87	266
dre-miR-459-3p	13	0.030	52	190
dre-miR-733-5p	85	< 0.001	344	129
dre-miR-735-3p	15	0.017	61	272
dre-miR-735-5p	14	0.017	59	468
chr9_46781	49	0.020	40	120
chr9_47479	19	0.013	55	95
dre-miR-27c-1-5p	24	0.003	101	84
dre-miR-187-3p	17	0.011	39	283
dre-miR-194a-5p	21	0.020	72	156
dre-miR-10d-5p	28	0.006	58	397
dre-miR-125a-2-3p	14	0.050	30	692
dre-miR-200a-3p	45	0.003	94	343
oni-miR-451b	10	0.046	32	19
dre-miR-144-5p	14	0.050	39	25
dre-let-7a-5-5p	18	0.026	63	67
ssa-miR-30d-2-3p	92	< 0.001	152	311
dre-miR-130a-5p	19	0.005	65	208
Control 32 °C vs control 28 °C: down-regulated				
chr4_36463	144	0.044	-21	379
hsa-miR-18a-3p	76	0.003	-24	386
mmu-miR-3963	539	0.050	-26	35
28 °C LPS vs 28 °C control: up-regulated				
dre-miR-25-5p	44	0.005	22	519
ssa-miR-30d-2-3p	51	0.023	15	311
28 °C LPS vs 28 °C control: down-regulated				
hsa-miR-3150b-5p	42	0.001	-31	62

494 **Table 4.** Enriched GO terms and KEGG pathways of differentially expressed mRNAs
495 predicted as targets of differentially expressed miRNAs in the 32 °C vs 28 °C incubation
496 temperature groups comparison. Fisher exact test, p-value < 0.05. BP: biological
497 process.
498

499

Category	Term	EnrichFold	P value
Up-regulated			
BP	GO:0007165~signal transduction	3.2	0.049
Down-regulated			
BP	GO:0010043~response to zinc ion	41.4	0.046
BP	GO:0044765~single-organism transport	2.1	0.031
BP	GO:0055085~transmembrane transport	2.7	0.038
KEGG	dre01100:Metabolic pathways	2.0	0.036

500 # Figure legends

501 **Figure 1.** Workflow of the data analysis. Raw reads were adapter-trimmed, and low-
502 quality reads were removed. Clean reads were sequentially mapped to mature miRNAs
503 from zebrafish, another 15 teleosts and three model species on miRBase. After
504 removing other types of non-coding RNAs, the remaining reads were used for
505 identifying novel miRNAs. All aligned reads were quantified and used for finding
506 differentially expressed miRNAs between temperature and treatment groups. The
507 target mRNAs of differentially expressed miRNAs were predicted and enriched GO
508 biological processes and KEGG pathways were identified. The integrative analysis was
509 performed between differentially expressed miRNAs and target genes that were also
510 differentially expressed in our unpublished RNA-seq data set [15].
511
512 **Figure 2.** (A) Read length distribution and (B) proportion of different types of non-
513 coding RNAs in clean reads.
514
515 **Figure 3.** Top 10 most abundant miRNAs across all the temperature and treatment
516 groups.
517
518 **Figure 4.** Volcano plot of differentially expressed miRNAs. (A) 32 °C vs 28 °C and (B)
519 28 °C: LPS vs control. padj < 0.05, Benjamin-Hochberg method.
520
521 **Figure 5.** (A) Representative enriched GO biological processes, and (B) KEGG and
522 Reactome pathways of target genes of differentially expressed miRNAs from the
523 comparisons i) control: 32 °C vs 28 °C and ii) 28 °C: LPS vs control. Fisher exact, p value
524 < 0.05. “Up-/Down-regulated” refers to the expression changes of putative mRNA
525 targets of differentially expressed miRNAs.
526
527 **Figure 6.** Venn diagram of predicted target genes and differentially expressed genes.
528 (A) 32 °C vs 28 °C and (B) 28 °C: LPS vs control.
529
530 **Figure 7.** Regulatory network of differentially expressed miRNAs and their target
531 genes. (A) 32 °C vs 28 °C and (B) 28 °C: LPS vs control. miRNAs are indicated by

triangles (yellow: up-regulated, blue: down-regulated), while target genes are indicated by ellipses. Significant correlations ($p < 0.05$) between miRNAs and target genes are in red.

536 ## Supplementary file legends

537 **Supplementary file 1.** Statistics of sequencing and mapping results in 30 libraries.

538 **Supplementary file 2.** Full list of novel miRNAs in each group.

539 **Supplementary file 3.** Full list of differentially expressed miRNAs. padj < 0.05,
540 Benjamin-Hochberg method.

541 **Supplementary file 4.** Predicted target genes of differentially expressed miRNAs.

542 **Supplementary file 5.** Enriched GO terms and KEGG pathways of target genes.

543 **Supplementary file 6.** miRNA and target gene pairs and associated correlations.

544

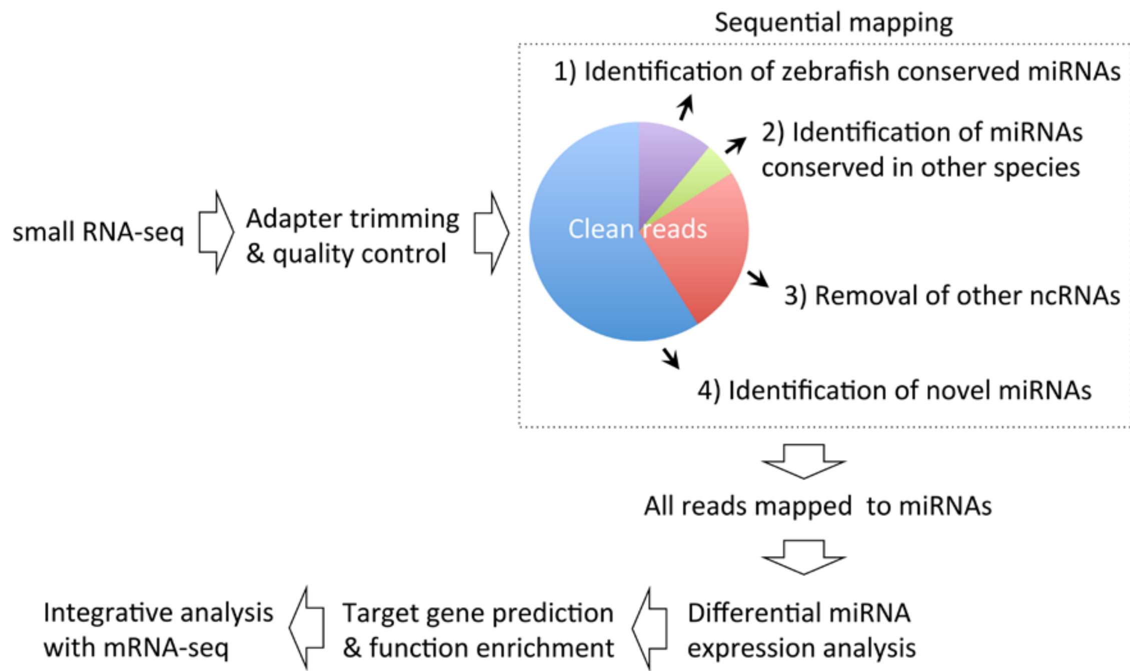
545 Supplementary files 1 - 6: http://jmofernandes.com/qirui.html

546

547 **Figure 1**

548

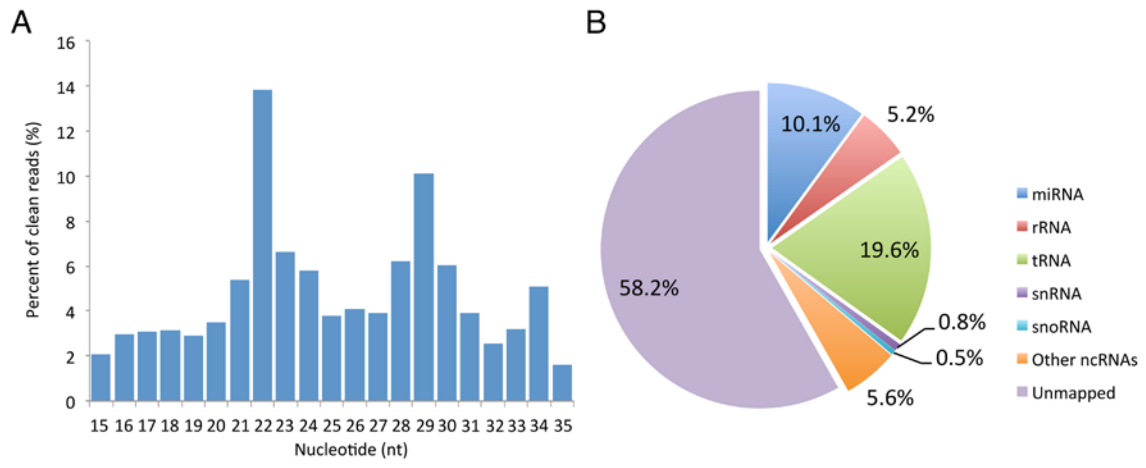
549



550 **Figure 2**

551

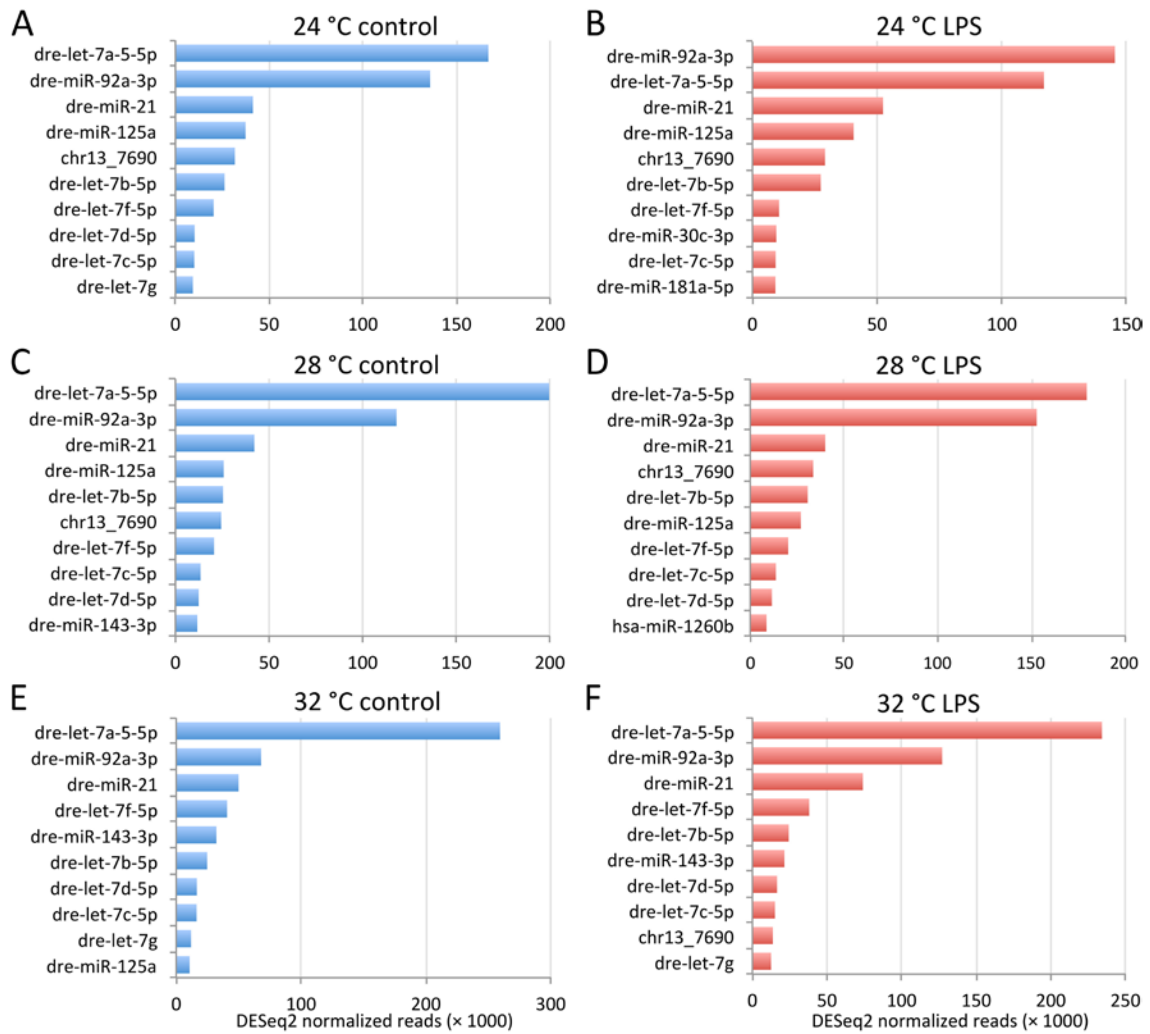
552



553 **Figure 3**

554

555



556

557

558

Figure 4

A

Contorl: 32 °C vs 28 °C

B

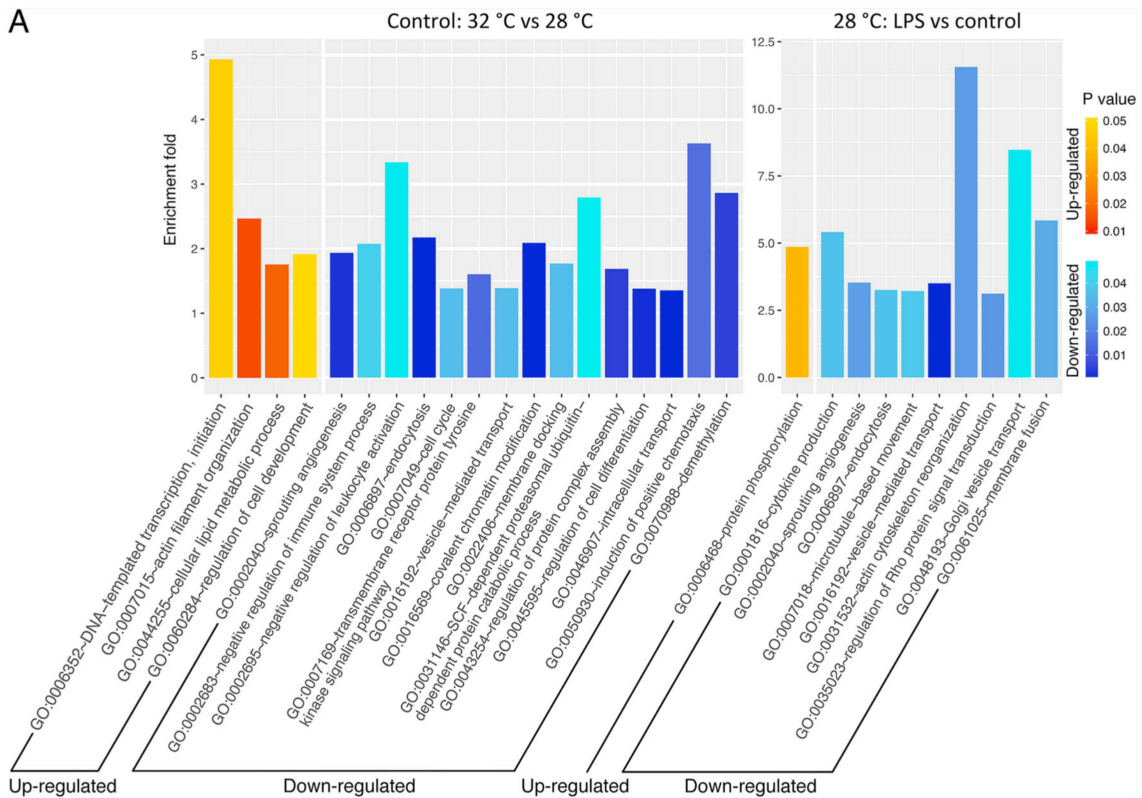
28 °C: LPS vs control

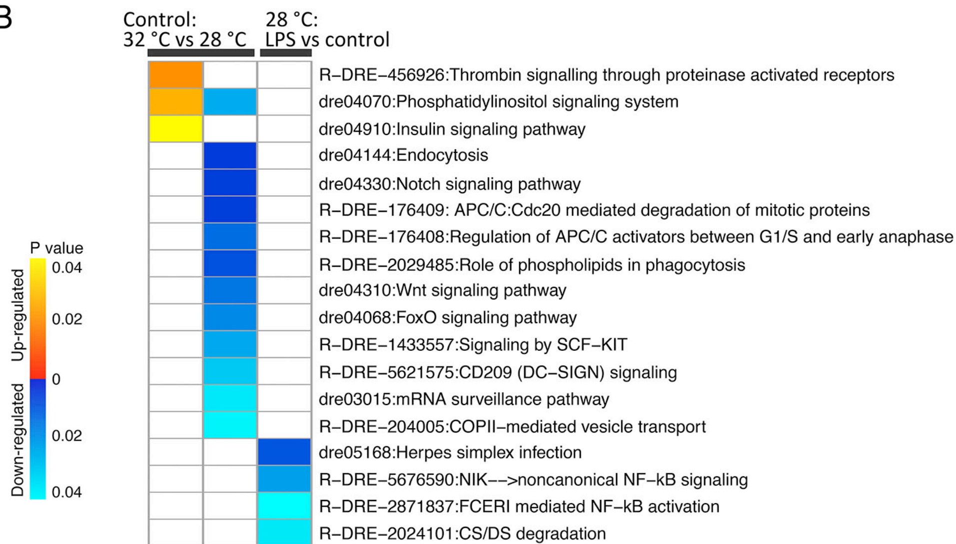
559

560

561

Figure 5

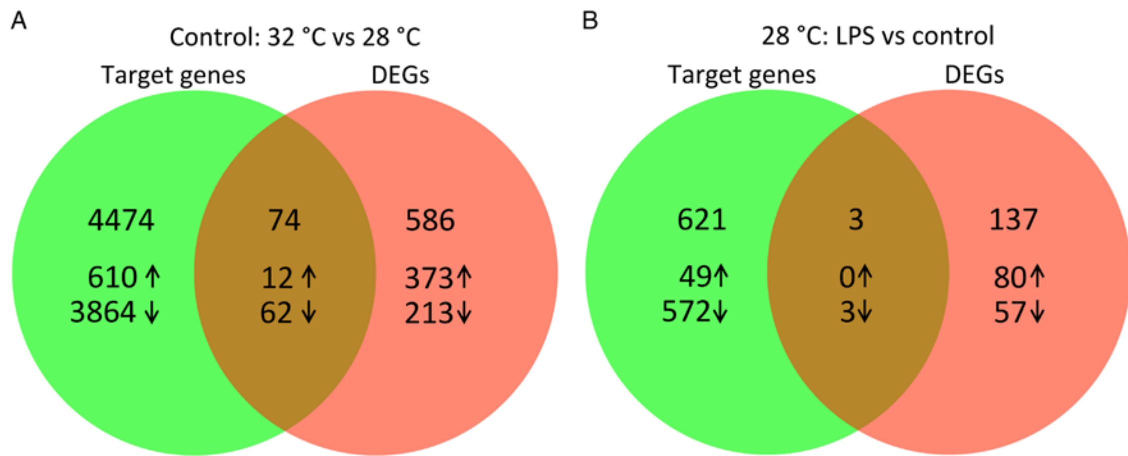




562 **Figure 6**

563

564



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566

Figure 7