Original Article

Seasonal Changes of Phagocytic Activity Enhanced by an Acoholic Mint Extract in Cultured Rainbow Trout

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ABSTRACT

Objective: We hypothesized that leukocyte numbers and their in vitro phagocytic activity in farmed rainbow trout are subject to negative influence of extreme temperatures for fish and that mint extract could alleviate these effects. **Materials and Methods:** Blood samples from farmed rainbow trout collected during winter and summer were subjected to leukocyte subpopulation counts (%) and carbon particle inclusion test to estimate the *in vitro* phagocytic activity after 15 and 30 min of incubation at 4 and 22°C, with or without alcoholic mint extract (In). **Results and Discussion:** There was a significant increase in lymphocytes ($p<0.05$) and monocytes ($p<0.01$) in summer versus winter, while the heterophils were dominant during winter ($p<0.01$). In vitro spontaneous phagocytosis increased with the seasonal increase in temperature (0.425 ± 0.094, winter and 0.835 ± 0.102, summer, $p<0.05$) and during the first reading period (0 to 15 min, $p<0.01$). The mint extract increased phagocytosis for the overall reading period from 2x (winter, 0.281 ± 0.221) up to 5x (summer, 1.032 ± 0.221) ($p<0.01$). **Conclusion:** The positive activity of the alcoholic mint extract during phagocytosis, more intense during summer than during winter, proved to be a temperature driven process, plant active principles playing a dual stimulating role for both monocytes and heterophiles.

Key words: Rainbow trout, Season, Leukocytes, Phagocytosis, Mint extract.

INTRODUCTION

Fish are some of the most primitive vertebrates and for the development of the immune system represent an important link between invertebrates and evolved vertebrates.[1] In phylogeny, the fish are the first to show both innate and adaptive cellular and humoral responses, capable to respond to microorganisms, toxins or malignant cells. These two systems work together to destroy invaders or to trigger defence processes.[2-4] Fish non-specific defence mechanisms are like those of other vertebrates, the phagocytic activity being fulfilled by heterophiles and macrophages.[5] The innate immune response is defined by lack of specificity towards antigens, thus being independent on previous contacts with those. Various non-self-particles can trigger the innate cellular and molecular effectors as the first line of defence in very short time scale.[6-7] The anatomical and functional integrity of the immunological defence mechanisms are extremely important and depend on the dynamics of host-aggressor interrelations.[8] The magnitude of the fish immune response is influenced by their habitat and its intrinsic factors, but also by their poikilothermic condition.[5]

Immunostimulants such as bacterial derivatives, animal and plant extracts increase resistance to infectious disease by enhancing non-specific immune defence mechanisms.[9]

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Meanwhile, numerous medicinal plants or their parts, single or in combinations, as traditional preparations or commercial products have been tested in diverse aquatic animals.[10]

Natural stimulating compounds are sought after by immunologists as cheap and readily available alternatives for different species of farmed animals, including fish, therefore medicinal plants could represent an appropriate source. The aim of the study was to investigate seasonal variation of phagocytosis as an indicator of innate immunity and the *in vitro* potential of an alcoholic mint (*Menta pipperita*) extract in enhancing phagocytosis in cultured rainbow trout (*Oncorhynchus mykiss*).

MATERIAL AND METHODS

Blood was sampled on heparine (50 IU/ml) by caudal vein puncture from conventionally farmed rainbow trout during winter and summer seasons and subjected to leukocyte subpopulation counts (Dia-Quick Panoptic method, CliniLab, Romania) and carbon particle inclusion test[11] to estimate the *in vitro* phagocytic activity after 15 and 30 min of incubation both at 4 and 22°C. The phagocytosis variants were represented by 0.5 ml of blood either un-supplemented (control), or added with 20 µl of 70° alcohol (solvent control) or 20 µl of an alcoholic mint extract (experimental variant), respectively. Phagocytic cells engulf inert particles such as carbon due to the defensive capacity of these cells. Two microliters of supernatant of India ink, obtained by centrifugation at 6000 rpm for 45 min were added to each variant. 150 µl of the mixture were transferred immediately to 2 ml of saline, and the rest was incubated for a total of 30 min. Similar aliquots of each variant were removed to 2 ml of saline every 15 min. The final tubes, containing saline, India ink and blood mixtures were centrifuged at 800 rpm and the supernatants were read spectrophotometrically at a wavelength of 535 nm; d=1 cm (SUMAL PE2, Carl Zeiss, Jena). There was a decrease in absorbance with time, as carbon was phagocytized. The phagocytic activity index was red in optical density units (ODU) and then expressed as the difference between the natural logarithms (ln) of the optical densities of the phagocytosis at 0-15 min, 15-30 and 0-30 min divided by time. Student's t-test was used to evaluate the statistical significance of the differences between the seasons and variants.

RESULTS AND DISCUSSION

In fish, nonspecific immunity is the first line of defence and is a considerable part of the immune response. Certain parameters of innate immune response, such as intensive respiratory burst, lysozyme activity, complement concentration and total IgM, are associated with disease resistance.[12] Non-specific immunity, similar to that of evolved vertebrates, depends on the activity of monocytes/macrophages, melanoma-macrophage centers, granulocytes and platelets.[13] Research in teleost fish described host defence strategies that involved intracellular (nitric oxide, phagolysosome fusion) and extracellular (heterophile degranulation and extracellular traps) that play an important role in resistance to disease.[14] The most important cells involved in non-specific defence are the phagocytes.[6-7] Macrophages and neutrophil granulocytes are the two types of cells with significant phagocytic capacity in rainbow trout.[15] They are the first cells to encounter invading microorganisms and therefore play a very important role in the initial stage of infection.[2]

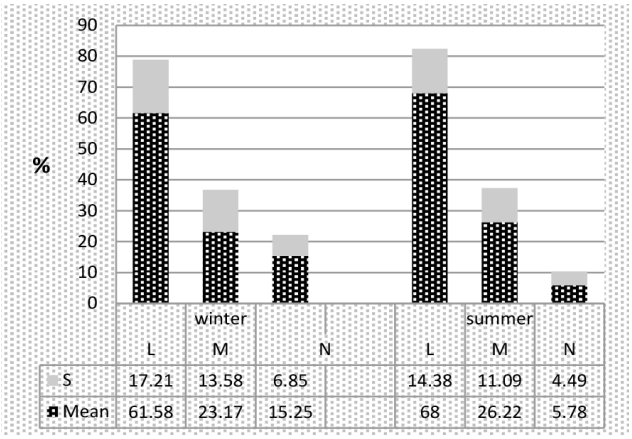


Figure 1: Leukocyte subpopulations in the two group of trout (winter and summer samplings).

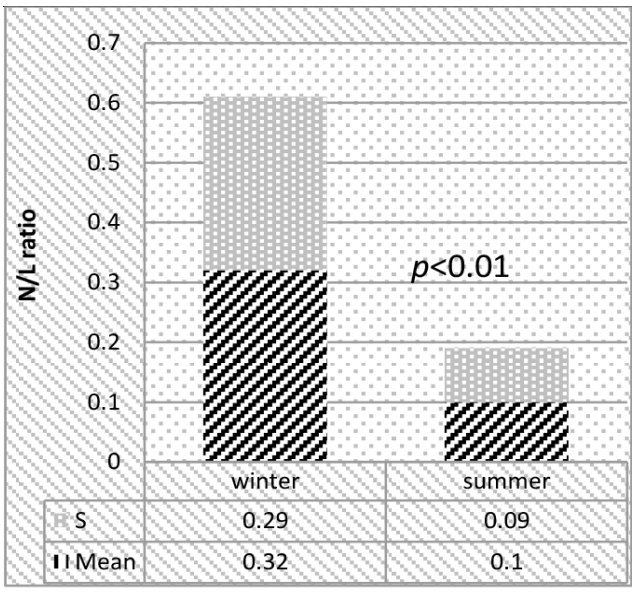
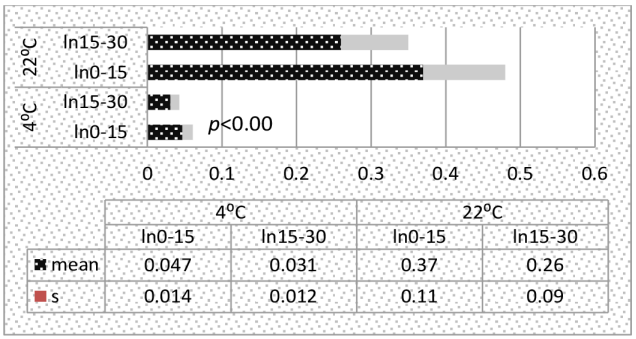


Figure 2: N/L ratio as a stress indicator: a more pronounced stress level was recorded during winter.



	4°C		22°C	
	In0-15	In15-30	In0-15	In15-30
mean	0.047	0.031	0.37	0.26
s	0.014	0.012	0.11	0.09

Figure 3: *In vitro* phagocytosis in farmed rainbow trout as influenced by the incubation temperature (x±s).

It has been reported that ambient temperature influences both antibody production and resistance to infection in cold blooded animals.[16]

The results obtained in this study indicated an increase of the lymphocyte and monocytes levels in summer versus winter, while the heterophils were dominant during the winter (Figure 1). Both the seasonal variance of the environmental temperature (Figure 2, and 3) and the incubation temperature during *in vitro* evaluation of phagocytosis statistically significantly influenced the

Table 1: Phagocytic activity in farmed rainbow trout during the winter season, under alcoholic mint extract influence (x±s).

Winter	Variants								
	Control			Alcohol			Mint extract		
	In0-In15	In15-In30	In0-In30	In0-In15	In15-In30	In0-In30	In0-In15	In15-In30	In0-In30
Mean	0.425	0.117	0.445	0.014	0.049	0.025	0.174	0.107	0.281
S	0.304	0.067	0.120	0.009	0.033	0.015	0.121	0.051	0.081

Table 2: Phagocytic activity in summer season in farmed rainbow trout. Alcoholic mint extract influence *in vitro* (x±s).

Summer	Variants								
	Control			Alcohol			Mint extract		
	In0-In15	In15-In30	In0-In30	In0-In15	In15-In30	In0-In30	In0-In15	In15-In30	In0-In30
Mean	0.835	0.261	0.574	0.721	0.272	0.993	0.804	0.227	1.032
S	0.994	0.767	0.460	0.321	0.543	0.475	0.431	0.521	0.221

N/L stress index, higher in winter than during summer and lowered the phagocytosis at 4°C.

Phagocytic activity is an important immunological parameter to define in investigating the bacterial infections in fish, since it is related to the nutritional status, age and breed of the animal, as well as prevention and therapies. Vegetal extracts can exert stimulating effects on immunocompetent cells.[7] Under the conditions of the experiment, the extract proved to be stimulating, suggesting the possibility of its use in improving immunity in farmed rainbow trout (Table 1 and 2).

The alcoholic mint extract improved phagocytosis from 2x (winter, 0.281±0.081) up to 5x (summer, 1.032±0.221) for the overall reading period (0 to 30 min) ($p<0.001$).

CONCLUSION

The changes in the leukocyte numbers differed depending on the temperature of the season and subpopulation. The phagocytosis stimulating activity of the alcoholic mint extract proved to be a temperature driven process due to the active principles from mint, which probably stimulated both the monocytes and the heterophiles, more intensely during summer than during winter.

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CONFLICT OF INTEREST

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

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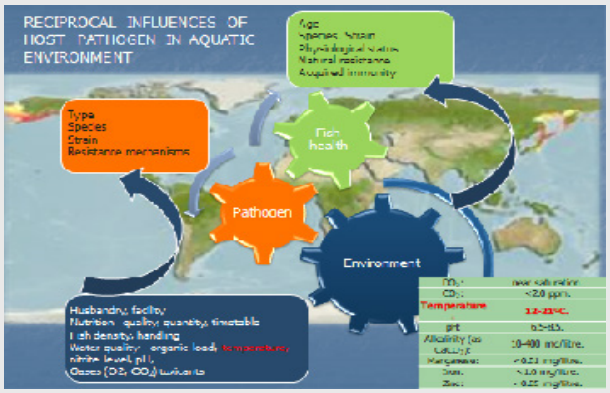
PICTORIAL ABSTRACT



SUMMARY

- This study investigated the effect of seasonal variation of environmental temperature on numbers and phagocytic function of leucocytes and the potential modulating influence of an alcoholic mint extract on those
- Leukocyte subpopulations were counted and N/L ratios calculated, while a carbon particle inclusion test was used to evaluate the phagocytosis.
- Lymphocyte subpopulations reacted differently to seasonal changes of the environmental temperatures.
- The active principles from mint probably stimulated both the monocytes and the heterophiles more intensely during summer than during winter, thus enhancing phagocytosis.

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