Effect of seasonal changes on the innate immunity of wild pseudois nayaur: potential reason for its endangerment

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Effect of seasonal changes on the innate immunity of wild *pseudois nayaur*: potential reason for its endangerment

B. Song, B. Wu, Seasonal changes impose harm to *pseudois nayaur*

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Abstract

It has been widely concerned that seasonal climate change may impose stress on mammal’s immune system, but this has never been connected to wildlife endangerment reasons. This study focuses on *pseudois nayaur*, a rare Chinese sheep facing a severe situation of disappearing. Wild *pseudois nayaur*s in Sichuan, China were observed in this research by an integrated method including habitat environment, foraging rules, and breeding activities investigation in ecology filed, accompanied with immunity research by PB (peripheral blood), including erythrocyte, neutrophils, NK(natural kill) cells, and macrophages immunity by means of erythrocyte rosette, phagocytosis, LDH (lactate dehydrogenase) release and ANAE (acid \(\alpha\)-naphthyl acetate esterase) method, respectively. The results show that *pseudois nayaur*s innate
and red blood cell immune functions were in degradation in autumn and winter, which were speculated to be the results of energy reallocation between reproduction and the immune system. In addition, the environment pressure found in Zhu Balong, Sichuan, China including food reduction, human disturbance, low temperature, and severe drought still existed, which may prevent the *pseudois nayaur* from normally immunity enhancement before winter. In conclusion, the innate immunosuppression found in this study especially at their breeding season in autumn and winter may be the ignored cause of wild *pseudois nayaur* endangerment and should be considered in their protection strategy.

Key words: *Pseudois nayaur*, ecological immunity, immunosuppression, seasonal change, reproductive coercion

INTRODUCTION

*Pseudois nayaur*, a cherished wild species in China under the protection of Chinese government by the law of State Protection of China and the International Union for Conservation of Nature (IUCN), is in danger from disappearing. From 1988 to 2003, their habitat area had decreased about 48,000 square kilometers in Batang, Sichuan. For the protection of this species, many studied their behavioral traits and living habits. For instance, some mentioned that the group behaviors of them were too vulnerable to escape from the predators (Liu et al., 2011; Shen et al., 2008). Moreover, the skin-based trade was revealed by researches and the *pseudois nayaur* were always easily caught by both human and predators because of the changeless water-drinking places (Long et al., 2008; Shen et al., 2008). As the protection law executed strictly, these predictable factors had been removed. However, the number of the *pseudois nayaur* were not increased as expected, only 500 ~ 700 ones were
speculated to be exist in this area (Hu, 2007), which indicates that the main reasons for its endangerment are still unknown and unresolved, which asks for more attention to its physiology features, especially for the immune system that depends individuals’ death directly.

Animal ecological immunology was defined in the 1990s, covering the study of animal immunity changes under certain environment conditions which were used to wide-ranged science research from physiology to molecular biology (Rolff and Sivajothy, 2003; Sheldon and Verhulst, 1996). One of the most important phenomenons to wildlife in the field is the periodic changes in immune system controlled by hormone and innate immunity. It was found that some mammals tend to enhance their innate immunity to get through the living challenges in winter (Martin et al., 2008), which is mainly correlated to temperature-controlled gene regulation (Xaquin et al., 2015). However, Harshman and Zera (2006) studied that a decrease of immunity also existed when the reproduction time is coming up, which means there may be a trade-off relationship between immunity and reproduction (Amdam et al., 2005; Fedorka et al., 2001), but few studies have targeted so far to the ones whose reproductive time is between autumn and winter which also ask for energy in immunity enhancement. So, what’s the body chioce when facing both chanllenges in reproduction and immunity? Through pseudois nayaur, the remained questions can be answered, and the relationship between its endangerment and this body choice could also be revealed.

In order to understand the trade-off relationship between immunity and reproduction in winter-reproduction mammals, and help protect pseudois nayaur in a more scientific way. The study investigated pseudois nayaur by the ecological immunology method. Seasonal changes of the immunity were studied by their peripheral blood, mainly reflected by innate and RBC immunity. Meanwhile, habitat environment, foraging rules were studied to evaluate the environment pressure, and the breeding
activities were observed additionally to be combined with immunity research and understand the trade-off choices that the body have made.

MATERIALS AND METHODS

Experimental animals and ecological research

Zhu Balong Nature Reserve, Sichuan, China was chosen to be the research area. Thirteen *pseudois nayaur* which were females and healthy were captured first and given a GPS-position collar. Every season (May, August, November, February in the next year), the same individuals were caught again without neutering for the peripheral blood (anticoagulated and colded in ice bag with temperature 0-4°C) immediately before transferred to a laboratory nearby in 24 hours, the total number of samples is 156, 3 replicates × 13 individuals × 4 seasons) from the vena jugularis externa. The manipulating process of animals were supervised by Animal Welfare Committee and Tangjiahe Government under the guidance of the Law of the People's Republic of China on the Protection of Wildlife, 2016, and the blood storage process follows the rules of International committee standard of hematology, ICSH,1993, using EDTA-2K. Their living habits including foraging rules, reproductive and clustering activities were also trailed through the GPS collar and faeces, before sighted.

Immunological examination

**Blood routine test.** The prepared peripheral blood transferred to the laboratory was used for routine blood test by the animal HA (hematology analyzer) (RR-29103302, Mindray), included indexes are WBC (White blood cell), Mon (Monocytes), Lymph (Lymphocyte), Lymph% (Lymphocyte%), Gran% (Granulocyte%), RBC (Red blood
cell), HCT (Hematocrit), MCV (Mean corpusular volume), MCH (Mean corpusular hemoglobin), RDW (Red blood cell volume distribution width), MCHC (Mean corpusular hemoglobin concentration), PLT (Platelet count), PCT (Platelet count), MPV (Mean platelet volume), HGB (Hemoglobin), PDW (Platelet distribution width) and Mon% (Monocytes). Every data conducted then were recorded and analyzed to see the differences between seasons.

**Blood smear analysis.** The blood smear was made according to the methods used by Ndao et al. (2004). Peripheral blood slides were made firstly, followed by Wright's-Giemsa solution (G1020, solarbio) Staining. Then, the stained slides were pictured through microscope (MF53, Mshot), after which WBC, and RBC were calculated by image-pro plus. (HGB can also be reflected by the measurement of light-stained area in BC)

**Detection of the erythrocyte immunity.** The erythrocyte rosette rate was measured by the method which was introduced by Chu et al. (2015). In brief, 4ml of yeast solution was taken, and an equal amount of serum was added to make 1×10⁸/ml concentration of bacterial suspension. Then, the red blood cell suspension was prepared and mixed to make the blood smear. After staining with Wright's-Giemsa, they were detected on a microscopic level. One hundred erythrocytes in each season were calculated. The Erythrocyte rosette rate=Number of red blood cells forming rosette/Total number.

**Detection of the immune function of neutrophils.** Under the condition of sterile operation, 5 drops diluted staphylococcus aureus was added into 1 ml anticoagulant, mixed and placed in carbon dioxide incubator at 37°C for 30 min, and shaked every 10 minutes. Then, the solution was used to make blood smears, before dried and observed with the microscope (MF53, Mshot). In each season, 100 neutrophils of every individuals were counted, after which the staphylococcus aureus-contained-neutrophils were recorded. The immune function of neutrophils was
reflected by this way, and the Phagocyte Rate= the number of staphylococcus aureus-contained-neutrophils in every 100 neutrophils.

**Determination of the NK cell immunological killing function by LDH.** LDH (lactate dehydrogenase) release method was used in the study to the detection of NK cell immunological killing function, as described by Konjević et al. (1997). Briefly, 0.1ml of the prepared NK cells of *pseudois nayaur* (taken in peripheral blood) and 0.1ml of the K562 cells (K562 cells were cultured for 28 hours in logarithmic phase and washed twice with RPMI-1640 medium. Then, they were suspended in 10% FCS-RPMI-1640 medium and detected by 0.5% trypan blue staining. Finally, the concentration was adjusted to 1×105/ml.) were taken. Then, among the experimental group, target cell natural release group (0.1 ml target cell + 0.1 ml 10% FCS-RPMI-1640 medium) and maximum release group (0.1 ml target cell + 0.1 ml 1% NP40 solution) were set up. After adding LDH substrate liquid for 15 min, the value A was measured by Enzyme Linked Monitor (ELx800, Bio-Tek, USA) under 570nm. NK cell activity %=[(Value A of the experimental group-Value A of natural release control group)/(Value A of maximum release control group)]×100%.

**Detection of the phagocytosis of macrophages.** Each blood smear slide of four seasons was prepared in the same way as mentioned above. After that, they were fixed with the solution of α-nae for 15min, then washed with distilled water for 5min and dried under 25 ℃. Then, the alpha NAE incubation solution was added, before put the smears into the humid cabinet at 37 ℃(avoiding light to incubate for 1 hour). To the next, after cleaning with water, the solution of methyl green dye was added to the droplet and then restained with water, after which the positive rate of macrophages was calculated under the microscope (MF53, Mshot) by the image-pro plus. (All reagents were purchased from the Alpha - naphthalene acetic acid assay kit, CAT#G2390 , Solarbio, Japan).

**Statistical analysis**
The data collected from PB experiments mentioned above were analyzed with t-test, one-way ANOVA. The data passed the homogeneity test for variance went through the LSD (Least-Significant Difference) test, and others were compared by Tamhane’s T2 test to keep the findings about the impact of seasons on *pseudois nayauls*’ immunity in accuracy. For the calculation process, it was done by SPSS20, and the outcomes was displayed with means ± standard deviation (\( \bar{X} \pm SD \)), P value, df, F, and MS figures.

**RESULTS**

**Ecological results**

**Research area.** The *pseudois nayauls* lived in Zhubalong preserve with an altitude between 2,600 and 3,500 meters. This area was a mountain valley landform and locates in the southeastern edge of the Qinghai-Tibet plateau. It was found that a large number of *pseudois nayauls* prefer staying at the places with cliff to be able to escape from their natural enemies. In addition, the climate here was obviously divided into dry season (from October to the next April) and rainy season (from May to September) every year, which makes winter harder to get through. To cope with the unfriendly climate, the concave hillside was used by them, which was both helpful for the cover under the rain and the warming up under the sunshine. For instance, in summer, the *pseudois nayaur* would move their sleeping place to a cool place to avoid the high temperature, strong sunshine, or rain. In winter, they tended to choose steep slopes under the sun as resting points.

**Foraging rules.** In this research, we observed that the foraging time of the *pseudois nayauls* was mainly on the morning and evening. Meadows, thickets, rocky escarpments with sparse grasses, and tall oak forests were the usual foraging sites. It was found that the main vegetation coverage was spiny roses and annual herbs, which accorded with the characteristics of vegetation coverage in dry-hot valley climate. However, the amount of the available food for *pseudois nayauls* was not in lacking.
Dry environments and low temperatures limited the growth of vegetation, besides, grazing activities led by human were also found, which imposed threats to their living conditions, especially in autumn and winter.

**Breeding activities.** In the aspect of breeding activities, we found that the time was mainly in November, which is at the boundary time of autumn and winter. Pregnant females give birth to offspring in May of the following year after about six months of gestation. Due to the harsh winter environment during pregnancy and the influence of natural enemies on the offspring, both the pregnancy success rate and the survival rate of the offspring were low, we speculated that this was an important reason why the species is in the danger of extinction and was ignored by most scholars.

**Cluster activities.** For group behaviours, the cluster types were observed in mixed group, separated female group and male group. The number of mixed groups was the largest, mostly 20 to 30 individuals per group. This might be the strategy of them to survival from predators and harsh climates, while it became harder and harder to keep that group number.

**Immunological detection**

**Blood routine.** The results of blood routine are shown in Table 1, 2, 3. For the immune system related cells figures in Table 1, there was no significant difference between seasons for WBC and Mon (p > 0.05). However, the number of Lymphs decreased rapidly in autumn, compared with the figures in spring (p < 0.01) and summer (p < 0.05), before climbed to the highest in winter (p < 0.01, compared with the figure in autumn). For the figure of Gran, it was seen a decrease in autumn compared with the figure in winter (p < 0.05).

To red blood cells related figures shown in Table 2, everyone except for RDW and MCV was significantly low in autumn and increased at different levels in winter. It should be noticed that MCH, MCHC, and HGB had apparently significant decreases
(p < 0.01) in autumn since spring, and then, recovered with an inadequate level in winter.

As for platelet related indexes, the figure for PLT was at the lowest level in autumn, compared to the highest one in winter (p < 0.01). In addition, the trend for the change of PDW was dramatic, it decreased considerably from spring to autumn (p < 0.01), which reached the bottom, and increased steadily at winter. For MPV, no evidence had shown the changes between seasons (p > 0.05), and there were both relatively low figures found in spring and autumn for PCT.

Overall, whether indexes in red blood cells, platelet, or immune related cells, wide-range decreases were found. Meanwhile, the recovery seemed to happen in winter but in a sign of weakness.

**Blood smear analysis.** The light stained part of red blood cells in autumn and winter was more obvious than that in spring and summer (least obvious), indicating that the hemoglobin contents in autumn and winter were lower in spring and summer, shown in the Table 4.

**Erythrocyte immunity.** The results is showed in Figure 2. The rosette rate of red blood cells decreased significantly in winter (P < 0.05) when compared with the figures in spring, summer, and autumn. The results showed that the immune function of erythrocytes was inhibited in winter.

**Immune function of neutrophils.** As showed in Figure 3 and Figure 4, seasonal changes had an significant impact on the number of neutrophils (P < 0.01). The phagocytosis rate of neutrophils in summer was significantly higher than that in winter (P < 0.01), and the indexes in summer and autumn were higher than those in spring and winter, respectively (P < 0.05). The results showed that the bactericidal function of neutrophils was inhibited in winter, and was the strongest in summer, followed by spring and autumn.
**Phagocytosis of macrophages.** As shown in Figure 5, there were significant differences between seasons (except for them between autumn and winter) of the figures ($P < 0.01$), and the figures were the lowest in autumn and winter. The results showed that the function of macrophages was inhibited in autumn and winter, but was relatively stronger in spring and especially in summer.

**NK cell immunological killing function.** As shown in Figure 6, seasonal changes had imposed great influence of the function for NK cells, they could only played the weakest role in autumn and winter, compared with that in spring and summer.

**DISCUSSION**

**Chanllenging living conditions**

In this research, four aspect of living conditions were studied, including habitat environment, foraging rules, breeding and cluster activities. Firstly, the places that *pseudois nayaur* lives in locate in the southeastern edge of the Qinghai-Tibet plateau, where mountain valley and cliffs are usually to be seen. It was found that they could take good advantages of the landform to escape from predators, and achieve the possibility of keeping safe from harmful sunshine and pour rain. However, step down to the bottom of the valley for water was unpreventable when the dry season (autumn and winter) was coming, which had been become the most risky movement of *pseudois nayaurs* because of the awaiting hunter traps and natural enemies. What’s more, although the food available for the *pseudois nayaur* was relatively enough in spring and summer, dry season had again made the vegetation in degradation especially for the higher but safer place. When facing such pressure, clusters got its own strategy, mainly expressed by acting together. As a result, more strong and healthy individuals would survive, but apparently, most females whose pregnancy period of time were from November to May are not among the group, which was proved by the low survival rate of cubs and the mothers found by Liu and Zhou (2011).
Trade-off between immune and reproduction of *pseudois nayaur*

In addition to the external challenges, the energy cost of reproduction and immunity in autumn and winter would also should be noticed. It has been found that, body usually make choices between reproduction and immunity, because the energy gained is limited. For most of the time, the immunity would be the suppressed one (Sussannah, 2007; Knowles et al., 2009; Deerenberg et al., 1997). This hypothesis can be the reason why *pseudois nayaur*’s immunity was found in a sigh of weakness in autumn. Among them, PLT and PCT, shown in Table 2, were at a low condition. Kuter and Rosenberg (1994) did a correlation analysis about the reason why PLT was low, the factors leading to the destruction of PLT in the experiment, such as diseases of the *pseudois nayaur*, were excluded. We believed that this phenomenon may be related to the reduction of megakaryocytic cells and growth factors in bone marrow, resulting in the weakened proliferation and differentiation of PLT, thus the function of wound healing was reduced. Then, MCH, MCHC, and HGB indexes showed seasonal suppression which indicated that malnutrition was exist and also suggested that the blood's ability to transport oxygen decreased obviously in autumn, and in this period *pseudois nayaur* may face to the risk of anaemia disorders (Cartwright and Lee, 2010; Fuchs et al., 2010). These findings also provided evidence of food shortages and the effects of overgrazing in the results. In terms of innate immune function, NK cells was inhibited, indicating the rising risk of infection, transformation or stress and decreased target cells kill function(Sun et al., 2011), even much higher probability of getting cancer (Albertsson et al., 2003). Moreover, The macrophages and neutrophils functions were inhibited, which meant the second line of immune defense decreased and bacterial resistance was declined (Gordon et al., 2010; Macmicking et al., 1997). In addition, erythrocyte rosette rate’s suppression in results was also a good proof of that, because the erythrocyte plays an important part in immune complex clearing, tumor, and innate immune (Cornacoff et al., 1983; Lutz, 2004).
**Winter immunity recovery**

In winter, the immunity suppression was also found but in a lower level. However, the food and water pressure were bigger than that in autumn, and the effects of low temperature were also beginning to emerge. So, the results were thought to be an evidence for the winter immunoenhancement hypothesis.

Through ecological investigation, it was found that cliffs and concave hillside were used by *pseudois nayaur* gaining appropriate sunshine, and it is believed that many small mammals have evolved a set of life-cycle strategies that can increase their immune function by using seasonal variation signals such as sunshine trait (length and strength) to improve their survival in winter (Sinclair and Lochmiller., 2000; Nelson and Demas., 1996). In this study, WBC was found the highest in winter and the upward trend was also obvious for the recovery of immunity in many other immunology findings, which are complied with similar phenomenon found previously in root voles. The rising number of WBC and other indexes would make them survive better in winter (Książek and Zub, 2014; Książek and Zub, 2017). For the reason of the primier rising of MCV in autumn might suggest the mechanism of resistance to the reduction of MCH and MCHC. For the further evidence to the phenomenon, studies in molecular level are needed. Although, the phenomenon of resistant immunosuppression was found, the future of the *pseudois nayaur* was not encouraging. After the trade-off choice been made in autumn to enhance the ability to reproduction in sacrificing of immunity, winter resistance seemed very important for their survival, and therefore, more efficient steps needs to be taken at this very season. The key point of its protection then turn to environment again, but in more specific to winter. As for human, overgrazing should be stopped firstly and the elimination of hunter trap should be done. To be more helpful, human interferance such as seeding the original vegeation are also supposed or desertification (Homewood and Rodgers, 1987) and wild *pseudois nayaur*’s extinction would be destination.
As a conclusion, we suggest that the habitat pressure of this species keeps increasing, and it is also found that these species have a large-scale immunosuppression when it is close to the special autumn breeding period in winter (Table 1), which confirms the trade-off between immunity and reproduction. At the same time, due to the challenge of winter climate, the immunity has not recovered quickly though there is a phenomenon of winter immunoenhancement. As shown in results, the survival of female *pseudois nayaur* during pregnancy was difficult. Therefore, we come to the conclusion that the immune suppression of *pseudois nayaur* is more threatening than other species. They are facing an immunosuppression for up to six months which is also the time for reproduction in population (Figure 7)

**Acknowledgments**

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**REFERENCES**


16. Kuter DJ, Rosenberg RD. Appearance of a megakaryocyte growth-promoting activity,


**Table 1. Immunity related cells’ blood routine results**

<table>
<thead>
<tr>
<th>Project</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
<th>df</th>
<th>F</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC(10⁹/L)</td>
<td>8.62±0.76</td>
<td>7.69±0.22</td>
<td>7.28±3.74</td>
<td>8.80±0.22</td>
<td>155</td>
<td>0.146</td>
<td>1.604</td>
</tr>
<tr>
<td>Mon(10⁹/L)</td>
<td>2.33±0.28</td>
<td>1.83±0.15</td>
<td>0.59±0.55</td>
<td>2.50±0.1</td>
<td>155</td>
<td>7.109</td>
<td>2.231</td>
</tr>
<tr>
<td>Lymph(10⁹/L)</td>
<td>8.17±1.53&lt;sup&gt;C&lt;/sup&gt;</td>
<td>7.13±0.46&lt;sup&gt;C&lt;/sup&gt;</td>
<td>2.57±1.21&lt;sup&gt;ABD&lt;/sup&gt;</td>
<td>9.27±0.40&lt;sup&gt;C&lt;/sup&gt;</td>
<td>155</td>
<td>8.243</td>
<td>25.983</td>
</tr>
<tr>
<td>Gran(10⁹/L)</td>
<td>7.57±0.58</td>
<td>6.84±0.16</td>
<td>6.47±0.22&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.62±0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>155</td>
<td>2.717</td>
<td>0.955</td>
</tr>
</tbody>
</table>

Data are expressed as mean standard deviation (sample number =156); A, B, C, D means different from those data in spring, summer, autumn and winter respectively. Capitalization means significant difference P<0.01, while lowercase represent extremely significant P<0.05. Overall, severe immunosuppression occurred in autumn, followed by winter, but in a resistant state.

**Table 2. Red blood cells related blood routine results**

<table>
<thead>
<tr>
<th>Project</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
<th>df</th>
<th>F</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC(10¹²/L)</td>
<td>5.86±0.49</td>
<td>5.94±0.07</td>
<td>3.30±1.62&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.34±0.36&lt;sup&gt;c&lt;/sup&gt;</td>
<td>155</td>
<td>2.580</td>
<td>5.807</td>
</tr>
<tr>
<td>HCT(%)</td>
<td>35.63±2.90</td>
<td>37.40±1.04</td>
<td>21.23±10.53&lt;sup&gt;c&lt;/sup&gt;</td>
<td>39.87±1.68&lt;sup&gt;d&lt;/sup&gt;</td>
<td>155</td>
<td>2.282</td>
<td>210.762</td>
</tr>
<tr>
<td>MCV(fL)</td>
<td>60.97±0.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.80±1.23</td>
<td>65.53±1.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>63.00±0.96</td>
<td>155</td>
<td>2.266</td>
<td>10.570</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>50.27±0.96&lt;sup&gt;C&lt;/sup&gt;</td>
<td>50.63±0.52&lt;sup&gt;CD&lt;/sup&gt;</td>
<td>24.00±0.17&lt;sup&gt;ABD&lt;/sup&gt;</td>
<td>48.33±0.37&lt;sup&gt;ABCD&lt;/sup&gt;</td>
<td>155</td>
<td>489.131</td>
<td>500.136</td>
</tr>
<tr>
<td>RDW(%)</td>
<td>13.23±0.55</td>
<td>13.47±0.98</td>
<td>16.17±1.84</td>
<td>13.47±1.05</td>
<td>155</td>
<td>1.346</td>
<td>5.823</td>
</tr>
<tr>
<td>MCHC(g/L)</td>
<td>826.00±7.55&lt;sup&gt;CD&lt;/sup&gt;</td>
<td>806.67±25.33&lt;sup&gt;C&lt;/sup&gt;</td>
<td>373.33±4.91&lt;sup&gt;ABD&lt;/sup&gt;</td>
<td>768.33±5.46&lt;sup&gt;AC&lt;/sup&gt;</td>
<td>155</td>
<td>245.296</td>
<td>138469.6</td>
</tr>
<tr>
<td>HGB(g/L)</td>
<td>294.33±22.67&lt;sup&gt;C&lt;/sup&gt;</td>
<td>302.67±0.67&lt;sup&gt;C&lt;/sup&gt;</td>
<td>117.33±6.06&lt;sup&gt;ABD&lt;/sup&gt;</td>
<td>306.67±15.01&lt;sup&gt;C&lt;/sup&gt;</td>
<td>155</td>
<td>43.668</td>
<td>25440.52</td>
</tr>
</tbody>
</table>

Data are expressed as mean standard deviation (sample number =156); A, B, C, D means different from those data in spring, summer, autumn and winter respectively. Capitalization means significant
difference $P<0.01$, while lowercase represent extremely significant $P<0.05$. Overall, severe immunosuppression occurred in autumn, followed by winter, but in a resistant state.

**Table 3. Platelet related blood routine results**

<table>
<thead>
<tr>
<th>Project</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
<th>df</th>
<th>F</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLT(%)</td>
<td>524.33±152.4 $^{4d}$</td>
<td>1016.33±215.48 $^{ac}$</td>
<td>396.67±117.32 $^{2bd}$</td>
<td>1132.00±56.08 $^{1c}$</td>
<td>155</td>
<td>6.028</td>
<td>391425.556</td>
</tr>
<tr>
<td>PCT(%)</td>
<td>0.25±0.07 $^{4d}$</td>
<td>0.52±0.09 $^{a}$</td>
<td>0.29±0.09 $^{d}$</td>
<td>0.57±0.03 $^{ae}$</td>
<td>155</td>
<td>4.746</td>
<td>0.078</td>
</tr>
<tr>
<td>MPV(FL)</td>
<td>4.87±0.23</td>
<td>4.83±0.09</td>
<td>5.37±0.37</td>
<td>5.00±0.10</td>
<td>155</td>
<td>1.136</td>
<td>0.179</td>
</tr>
<tr>
<td>PDW(FL)</td>
<td>16.33±0.33 $^{c}$</td>
<td>15.67±0.33 $^{C}$</td>
<td>12.63±0.59 $^{ABD}$</td>
<td>16.00±0.00 $^{c}$</td>
<td>155</td>
<td>20.286</td>
<td>8.723</td>
</tr>
</tbody>
</table>

Data are expressed as mean standard deviation (sample number =156); A, B, C, D means different from those data in spring, summer, autumn and winter respectively. Capitalization means significant difference $P<0.01$, while lowercase represent extremely significant $P<0.05$. Overall, severe immunosuppression occurred in autumn, followed by winter, but in a resistant state.

**Table 4. Blood smear cell count analysis**

<table>
<thead>
<tr>
<th>Project</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
<th>df</th>
<th>F</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10$^9$/L)</td>
<td>8.58±0.76</td>
<td>7.51±0.22</td>
<td>7.27±3.74</td>
<td>8.86±0.22</td>
<td>155</td>
<td>0.165</td>
<td>1.625</td>
</tr>
<tr>
<td>RBC (10$^{12}$/L)</td>
<td>5.71±0.49</td>
<td>5.91±0.07</td>
<td>3.46±1.62 $^{d}$</td>
<td>6.82±0.36 $^{c}$</td>
<td>155</td>
<td>2.731</td>
<td>5.994</td>
</tr>
</tbody>
</table>

Data are expressed as mean standard deviation (sample number =156); A, B, C, D means different from those data in spring, summer, autumn and winter respectively. Capitalization means significant difference $P<0.01$, while lowercase represent extremely significant $P<0.05$. Table 4 shows that RBC cells decreased significantly in autumn which indicated the reproduction stress in that time. And increased in winter, which was thought to be relevant to the compensatory strategies.
Figure 1. Four seasons blood smear analysis diagram. Figure a, b, c and d are the blood smears in spring, summer, autumn and winter, respectively (x 1000, stained with Wright's-Giemsa staining). The light stained part of red blood cells in autumn and winter was more obvious than that in spring and summer, and the least obvious in summer, indicating that the hemoglobin content in autumn and winter was lower than in spring and summer, while the hemoglobin content in summer was the highest.

Figure 2. Statistical chart of the seasonal changes of the erythrocyte wreath rate. * in the figure represents a significant difference between the two groups (P < 0.05), and
** represents a very significant difference (P < 0.01). The comparison between each group and the winter group was conducted before pairwise comparison. The rosette rate of red blood cells significantly decreased in winter (P < 0.05) when compared with the spring, was low summer and autumn. The results showed that the immune function of erythrocytes was inhibited in winter.

Figure 3. Seasonal changes of staphylococcus aureus phagocyted by neutrophils. Figure a, b, c and d are the blood smears in spring, summer, autumn and winter, respectively (x 1000, stained with Wright's-Giemsa staining). Phagocytosis rate of neutrophils in summer was significantly higher than in winter (P < 0.01), the indexes in summer and autumn were higher than those in spring and winter, respectively (P < 0.05).
Figure 4. The statistical diagram of the seasonal changes of staphylococcus aureus phagocyted by neutrophils. * In the figure represents a significant difference between the two groups (P < 0.05), and ** represents a very significant difference (P < 0.01). The figure suggests that the function of neutrophils was effected by the seasonal changes. It is better in summer than in spring and autumn, while the suppression was found in winter.
**Figure 5.** Seasonal variation of macrophage positive rate. * In the figure represents a significant difference between the two groups (P < 0.05), and ** represents a very significant difference (P < 0.01). It can be concluded in the figure that the macrophage positive rate was also in change with the period of seasons, but it was in the autumn that the rate declined more significantly instead of winter, which was speculated to be relevant to the reproduction matter.
Figure 6. statistical diagram of the activity rate of NK cells in four seasons. * In the figure represents a significant difference between the two groups (P < 0.05), and ** represents a very significant difference (P < 0.01). NK cell activity rate was shown in an increase in summer which was consistent to above indexes, and the rate in spring and autumn, was familiar but declined in winter.
Figure 7. Eco-immune Pathway of Endangered of *pseudois nayaur*. The immunosuppression was caused by a varied way and mostly resulted from the seasonal changes and reproduction hobby. The harsh climates and human activities causes the immunosuppression together against the evolutionary strategies that *pseudois nayaur* owns to protect from the death, but are not as much effective as the challenges they are facing.