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Prevalence of the Amphibian Pathogen *Batrachochytrium dendrobatidis* in Endangered *Neurergus microspilotus* (Caudata: Salamandridae) in Kavat Stream, Western Iran

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Abstract: Histological evidence obtained from toe clips of free-living endangered *N. microspilotus* confirmed the presence of *Batrachochytrium dendrobatidis* in this newt. This inference is based on the presence of zoosporangia with discharge papilla embedded in the skin epithelium and projecting beyond the surface of the epithelium. Data gathered from lines of arrested growth (LAGs) were used to determine the age of the sampled newts. Of 81 sampled toe clips of the living *N. microspilotus* 48% tested positive for *B. dendrobatidis*. Although no clinical signs of disease were found in the sampled *N. microspilotus,* data gathered from histological evidences provided from toe clips allowed an estimate of the asymptomatic infection to *B. dendrobatidis* in different age groups. The prevalence rates over eight age groups ranged from 13% in four year olds to 81.9% in 10 years and older.

Key words: *Batrachochytrium dendrobatidis* · *Neurergus microspilotus* · Lines of Arrested Growth · Prevalence

INTRODUCTION

Recent evidence suggests that disease may be an important factor in the declines of certain amphibian populations [1-3]. Pathogens of different kinds may infect amphibians at various life stages and can be the immediate causes of mortality or can lead to sublethal damage such as severe developmental and physiological abnormalities [4]. The fungal disease chytridiomycosis has shown to have catastrophic effects on amphibian populations leading to declines and even extinctions in several species [5-7]. Several other factors have also been reported as globally threatening factors to the existence of amphibians. Therefore, it is possible that chytridiomycosis in conjunction with several other factors has had a synergetic impact on endangered amphibians. Other parameters that in combination with chytridiomycosis can cause population declines include habitat loss and degradation, UV-B radiation, contaminants in aquatic environments, exotic species, global climate change and other pathogens and diseases [6, 8].

Herpetologists often remove amphibian toe for mark-recapture studies to estimate population sizes [9], to estimate the age of individuals [10] and to assess the presence of *B. dendrobatidis* and other pathogenic agents in natural amphibian populations [11]. The idea that *B. dendrobatidis* only grows on keratinized skin has been developed by Olsen *et al.* [12]. *B. dendrobatidis* infects the keratinized epidermal cells causing a hyperkeratosis and hyperplasic response of the stratum corneum and stratum granulosum. Asymptomatic and non-fatal infections of Chytridiomycosis have been reported for many species of amphibians [13, 14]. There are many reports on mass mortalities in postmetamorphic amphibians infected with *B. dendrobatidis* [15-17] however, no mortality has been reported in infected anuran larvae. Although amphibians have been

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experimentally infected from laboratory reared *B. dendrobatidis* zoospores studies documenting transmission within and between life stages are lacking [18].

Although histopathologic assays seem to be less sensitive than recently developed molecular assays [18], most of the work on diagnosis of chytridiomycosis has been concentrated to microscopy to visualize sporangia in the skin of dead animals or toe clips of live specimens. Sensitivity of this examination is very good in heavily infected amphibians [19]. This method has been successfully used to document the presence of *B. dendrobatidis* in live and also cases of chytridiomycosis in dead yellow spotted mountain newts from Kavat Stream in western Iran [20]. Chytridiomycosis has been reported from Iran in the free living *N. microspilotus* and also in a captive breeding facility containing this newt [20]. In these dead specimens, immature and mature zoosporangia, zoospores and discharge tubes of chytrid fungus, *Batrachochytrium dendrobatidis* were detected. These evidences provided from finger toes confirmed the presence of *B. dendrobatidis* in the host keratinized epithelial cells of the skin tissue [20]. The present study aims to estimate the prevalence of this disease in the population of the yellow spotted mountain newt in Kavat stream by estimating the infection rate to *B. dendrobatidis* in different age groups.

MATERIALS AND METHODS

N. microspilotus has been reported in several first order streams in western Iran but in most of these streams, this newt is present at very low densities [21]. Only in Kavat Stream may a population of over several hundred be present [17]. Kavat Stream (34 52' N, 046 30' E) is a well-known habitat for *N.microspilotus.* It is a relatively long stream with a high level of average discharge of 625.7 l/s [21]. This site is in an area of relatively less disturbed open woodland and low-intensity agriculture with established horticulture practices along the stream. The major crop is walnuts and other fruit trees. The horticultural activities rely on an extensive system of terracing supported by stone walls and divergence of water from the main stream. Old and tall walnuts tree provide shade estimated visually to be cover 75% of the stream [22]. A large and permanent karst spring feeds the stream. In early spring, water from subterranean seepages around the stream may rise to feed several parallel shallow streams. At very steep banks of the stream, there are outcrops of sedimentary rock with high porosity making them valuable areas for the newts to forage in. Decaying leaf litter was present throughout much of the site. As an indication of cold climate appropriate for the yellow spotted newt, various species of mosses are present as part of benthic, epiliths and epipelic cover in the stream.

The specimens used in the present study were 81 toe clips obtained from 37 males and 44 females' adult *N. microspilotus* from Kavat Stream. The newts were all caught in the daytime of the 3rd of June 2011. The salamanders were examined for any clinical symptoms associated with chytridiomycosis. After photography, the longest finger (I.E. second or third) of the forelimb was cut and preserved in 10% formalin solution. The wound was disinfected with Betadine. Finally, the newts were released at their collecting sites. The sex of each mature individual was determined according to external secondary sexual characters: Male has a fleshy protuberance at the base of the tail, whereas female has a prominent cloaca but without the protuberance. Post-metamorphic subadults are differentiated from adults in having smaller body length and lacking both the protuberance and the prominent cloaca [23]. Since sampling took place in June when most females have just spawned, no individual age 0-1 was available in this study. Post-metamorphic sub-adults age 1-2 were also absent presumably because of their specific activity pattern which involved late appearance until maturity in their fourth year similar to *Mertensiella caucasica* [24] and *Tylototriton verrucosus* [25].

The phalange was washed in running water for about 24 hours then were decalcified in a solution of ethylene diamine tetra acetic acid (EDTA) for a week. Each phalange was dehydrated in a graded alcohol series, cleared in xylene and embedded in wax. Then each phalange was sectioned at 7-8 µm using a rotary microtome. Serial sections were made from each phalange, at mid-diaphyseal level, with the smallest marrow cavity and were placed four ribbons on each slide. The resulting sections were stained in Harris hematoxylin. The sections were mounted on slides and observed with a light microscope. Bone sections from each individual were photographed (At the same magnification) using a Dine capture, allowing for simultaneous comparison and facilitating the analysis of the presence of *B. dendrobatidis* and the lines of arrested growth (LAGs).

Chytrid infections can be diagnosed based on the presence of zoosporangia with discharge papilla embedded in the skin epithelium [19, 20]. The sections of each individual was separately examined and recorded as infected or not. Sections were also examined for growth

rings in the phalange bone sections. Each ring was considered equal to an annual layer in this species as has been reported for *Mertensiella caucasica* [24] and *Bufo viridis* [26]. Sections were examined using a stereoscopic microscope under 250X magnification. Finer layers were also examined with a compound microscope under higher magnification. If two of three readings were the same, that number was used as the age estimate. Small discrepancies resulting from artifacts in the slides were resolved by a fourth reading. Larger discrepancies were disregarded. Since the toe clips were sampled in June 2011 and at that time, no larvae were available in the stream, growth rings are restricted to post-metamorphic individuals. The 95% confidence interval any particular age is calculated as: 95% CI=P±1.96×SE; where P is the observed proportion and SE is the standard error of the true proportion (SE=v [P× (1-P)]/N).

RESULTS

Despite the unique histological symptoms of *B. dendrobatidis* (*Bd)*, we found no clinical signs of disease in 81 wild-caught amphibians. Sections of a stained and formalin fixed finger of an adult *N. microspilotus* is shown in Fig. 1. This figure demonstrates an epidermis infected with *B. dendrobatidis* with zoosporangiums with various degrees of development. In most cases, the chytrid fungus is associated with mild focal hyperkeratosis in the area of stratum corneum. The sporangia presented the size, shape and location characteristics of those of *B. dendrobatidis* (Figs. 1 and 2). In all sections prepared from toe clips with *B. dendrobatidis*, the early stage of *B. dendrobatidis* contains a central mass which is basophilic and roughly spherical or oval. The mass of sporangia is fairly homogenous in staining characteristics. The mature zoosporangium contains multiple discrete 2-3 µm basophilic spherical zoospores. Zoospores can be released via the discharge papilla. On some stained sections, the rhizoids are present. These rhizoids are thin root-like projections from the thallus and they usually measure 7-20 mm in histologic section.

Data gathered from growth rings of 81 toe clips from the endangered yellow spotted mountain newt (*N. microspilotus*) in Kavat Stream were used to determine the age of the sampled newts (Table 1).

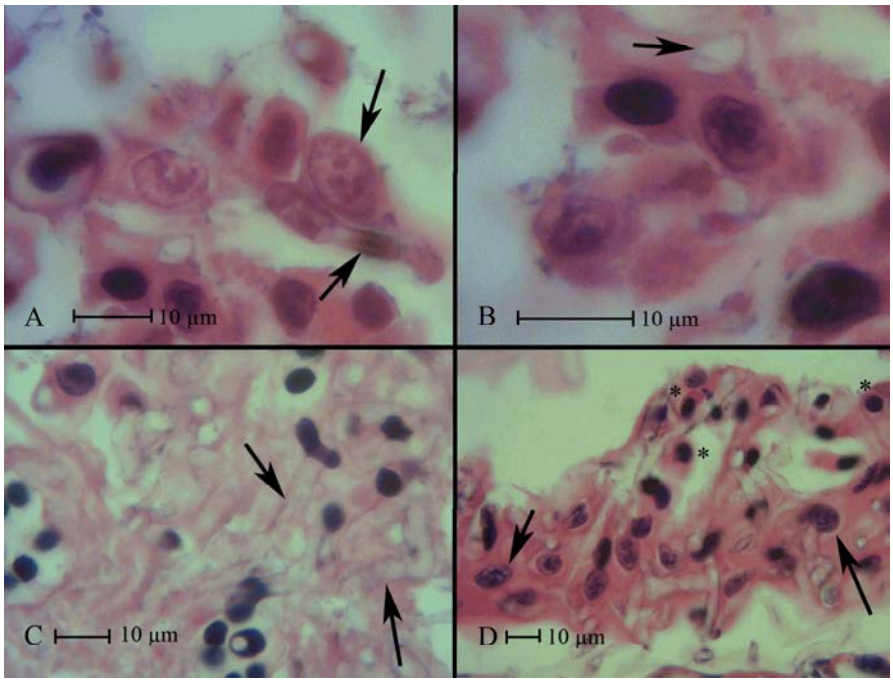

Fig. 1: Photomicrograph of epidermis infected with *Batrachochytrium dendrobatidis*
(A) Mature zoosporangium with discrete basophilic zoospores and a discharge tube (Arrow), (B) "Empty" thalli that have previously discharged its zoospores. The thalli in the center are "colonial" thalli as indicated by fine internal septation that appears to divide the thallus in half, (C) Developing Rhizoids with root-like projections from the tallus, (D) Developing multinucleated zoosporangium with basophilic zoospores (Arrow) and there also are several uninucleate developing thalli (*). (H and E staining). Bar = 10 µm.

Table 1: Number and percent of individuals in every age group, average prevalence (%) of infection with *B. dendrobatidis* and confidence interval (CI) in *N. microspilotus* collected from Kavat Stream, western Iran

Age (years)	Specimen No.	%Specimens	% infected by *Bd*	Confidence interval (CI)
3	8	9.88	45.5	11-80%
4	14	17.28	13	0-30.1%
5	8	9.88	34.12	1.3-70%
6	14	17.28	45.5	19.4-71.6%
7	13	17.28	21	0-43.1%
8	7	8.64	65	29.7-100%
9	7	8.64	78	47.29-100%
10+	10	11.11	81.9	58-100%

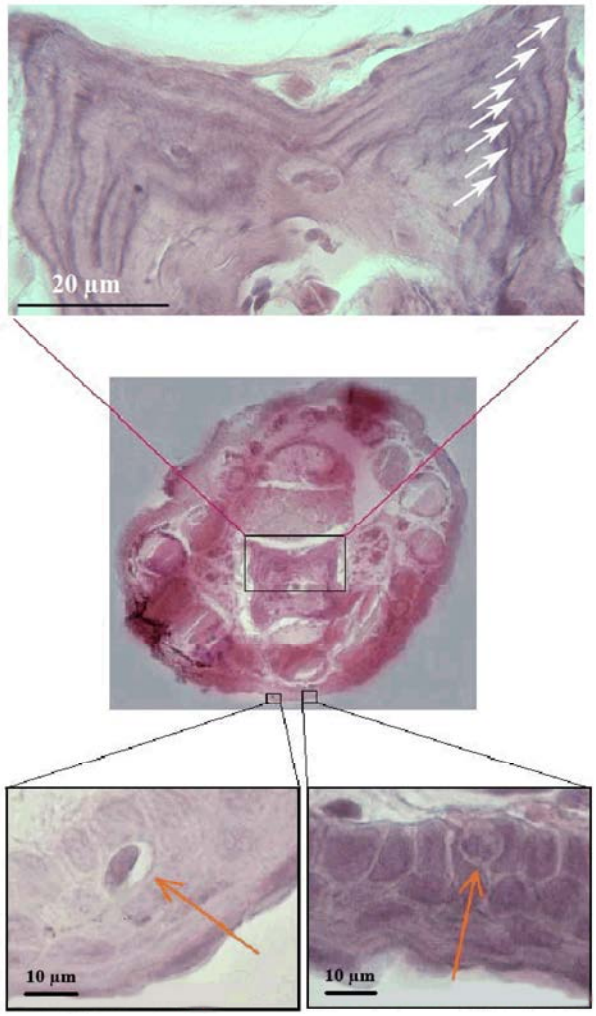

Fig. 2: Cross sections through phalanges of *N. microspilotus* in the upper slide. Seven lines of arrested growth (LAGs) are observed in the periosteal bone. In seven years' old (down) newt homogenous zoosporangium with basophilic immature zoospores are observed in the subsurface layer.

Age of the specimens was determined blankly by three observers (Author, 2, 3, 4). Based on the number of growth rings the yellow spotted newt has a high longevity of up to 13 Years. Average longevity for this newt is 6.44 years. Of the 81 samples from toe clips from live *N. microspilotus* 48% tested positive for *B. dendrobatidis*. The prevalence rates in different age groups are shown in Table 1. This rate ranges from 13% in four year olds to 81.9% in 10 years and older. While the prevalence rates are high in very young and very old individuals, the middle age groups had the lowest prevalence rates. Fig. 2 illustrates homogenous zoosporangium with basophilic immature zoospores in the subsurface layer in a slide with growth rings.

DISCUSSION

This study provides the first record of the extent of prevalence of chytridiomycosis in the endangered yellow spotted mountain newt from Iran. The occurrence of this disease has been previously reported by Parto *et al.* [20]. The intensity of the disease based on the number of zoosporangia found in the sections of toe clips was not high. Moreover, since this study is conducted on free living individuals, the results provide evidence on the extent of asymptomatic infection of chytridiomycosis. There are many reports of amphibians which carry the fungus sporangia but do not exhibit severe coetaneous lesions [27]. It is also possible that lightly infected individuals act as vectors while remaining healthy as there are reports of some amphibians which appear to be reservoirs for the fungus [28].

Our results are likely an underestimate of the true incidence of chytrid infection for *N. microspilotus* because our sampling may not truly represent the entire population of this species. *N. microspilotus* has been reported to occur in 14 first order streams in southern

Zagros Mountains but their abundance range from only few to several hundred individuals. In present study, the accuracy of the rates of the asymptomatic infection to *B. dendrobatidis* in different age groups in endangered *N. microspilotus* is associated to the sample size. Population size of *N. microspilotus* in the study area is not exactly known. Several estimates of relative abundance (Number of individuals observed per unit of length) yielded 600 [22] for the entire stream. In another attempt using a photographic identification method, mark-recapture techniques yielded 1200 individuals. The sample size of 85 probably represents over 7% of the population.

Chytridiomycosis can be diagnosed by routine Hematoxylin and Eosin stains without any specific fungal stains [19, 20]. Other techniques have been developed to diagnose chytridiomycosis in amphibians. For example, Berger *et al.* [19] used polyclonal antibodies to *Bd* for an immunoperoxidase stain to detect antigens of the fungi in the tissue sections. Such staining can be useful when identification of chytridiomycosis based on histological structure is doubtful. Clinical symptoms in live specimens include lethargy, in appetence, loss of digits, irregular skin sloughing, weight loss, accumulation of gelatinous material and fungal infections on the terminal two-thirds of the tail. Clinical evidence also appeared in several larvae reared in a captive breeding facility at Razi University.

The overall incidence of *B. dendrobatidis* infection in the yellow spotted newt in Kavat Stream was relatively high but not surprising. Compared to similar studies in free-living amphibians which may reach 100% [28] the present rate of infection in the yellow spotted mountain newt, is not very high. The prevalence of *B. dendrobatidis* over eight age groups (Table 1) is not evenly distributed. Of the 81 samples from toe clips from living *N. microspilotus*, 48% tested positive for *B. dendrobatidis*. The prevalence rates in different age groups are shown in Table 1. This rate ranges from 13% in four year olds to 81.9% in ten year olds and older. Perhaps this high variation in the prevalence of asymptomatic infection to *Bd* in different age groups in endangered *N. microspilotus* can be attributed to changes in immune resistance in various age groups. It is assumed that the body's immune resistance is low at the beginning of each individual's life while in the middle with the experience of various diseases, immune resistance is increased and again reduced in old age (Fig. 3).

Along with a change in the body's immune resistance associated with a similar variation in the prevalence of the disease another reason for the increased prevalence of infection to *Bd* in the older age groups could be the exposure to zoospores for a longer period. It has been demonstrated experimentally that some species of amphibians can experience the transmission of the disease via release of zoospores of *B. dendrobatidis* from infected amphibians into their environment. Such infected individual were able to cause a subsequent infection of uninfected amphibians by these zoospores [30, 31]. It is, therefore, possible that with increasing contact of individuals with zoospores existing in various parts of the aquatic environment such as substrate on the surface of streamside rocks or boulders in the consecutive years, infection may be increased [3, 32].

There are at least 21 amphibian species are reported from Iran [33] including some that are critically endangered by IUCN criteria. The yellow spotted mountain newt occurs in Iran and Iraq is listed as critically endangered by IUCN. This species occurs in different

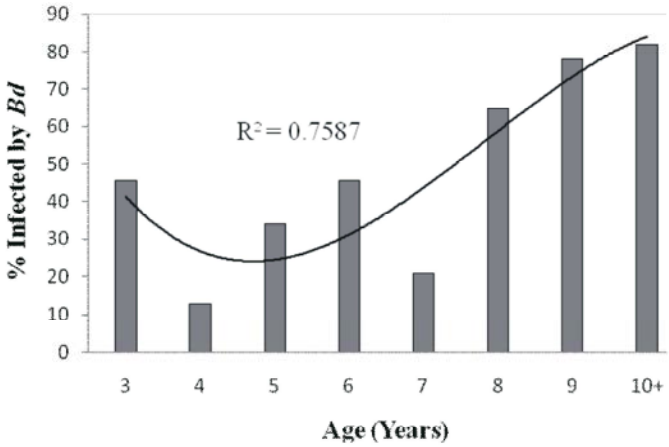

Fig. 3: The frequency distribution of the asymptomatic infection to *Batrachochytrium dendrobatidis* in different age groups in *N. microspilotus*

climatic regimes including warm and dry areas in eastern Iraq and also cold and wet areas at higher elevation in mid-Zagros Range in Iran. The Kavat Stream is located at particularly high elevation and with its cold and wet climate regime may be suitable for *Bd.* According to models predicting the global distribution of *Bd* based on environmental variables cold high altitude mountains are favorable for the infections [34]. Average temperature and relative humidity in this area are 16.5 and 78% respectively. Such climate seems to match with optimum temperature reported for *Bd* at 17 to 23°C with moist conditions [35].

Most studies use histopathological analysis or real-time PCR to determine the presence or absence of *Bd* at the level of individuals in a population [20]. Such studies are less informative than studies examining prevalence within populations and communities over time. It is also possible to focus on the ways the chytrid disease can be transferred between different species at community level. Some authors believe it is time to shift our attention to the question of population and community-level effects of this disease. Diagnosis of chytridiomycosis based on direct histological examination of skin tissue is not adequately sensitive and can have a high positive predictive value when used on heavy infected amphibians. However, chytridiomycosis is a common disease and can also be found in healthy individuals. Further testing is needed for additional species of amphibians throughout the country to assess potential threats.

Unlike many reports from tropical and subtropical wetlands, where *Bd* is associated with high mortalities of diverse amphibian communities [36-38] the present study documents a high prevalent occurrence of *Bd* with very low observed mortality. In present study no clinical symptoms such as lack of appetite, loss of weight, loss of digits, accumulation of gelatinous material on the skin and plain fungal infections was found in the sampled *N. microspilotus*. However, there are records of mortality associated with *B. dendrobatidis* in dead *N. microspilotus* from Kavat Stream [20]. In a similar study based on histological examination on *Neurergus kaiseri* a closely related species to *N. microspilotus* no evidences confirmed the presence of *Bd* in toe clips of free living newts. There are no reports of the extent of infection to *Bd* in other stream-breeding amphibians (7 species) in Iran which may be potentially susceptible to chytridiomycosis, including species with similar life-history traits and shared phylogenies. Information on the distribution and prevalence of *Bd* in both lentic and lotic freshwater amphibians in Iran is very sparse. However, such information is important because of its potential threat to the amphibian populations.

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REFERENCES

1. Blaustein, A.R. and J.M. Kiesecker, 2002. Complexity in conservation: lessons from the global decline of amphibian populations. Ecol. Let., 5: 597-608.
2. Daszak, P., A.A. Cunningham and A.D. Hyatt, 2003. Infectious disease and amphibian population declines. Divers. Distrib., 9: 141-150.
3. Lips, K.R., F. Brem, R. Brenes, J.D. Reeve, R.A. Alford, J. Voyles, C. Carey, L. Livo, A.P. Pessier and J.P. Collins, 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proc. Natl. Acad. Sci. USA., 103: 3165-3170.
4. Voyles, J., E.B. Rosenblum and L. Berger, 2011. Interactions between *Batrachochytrium dendrobatidis* and immunity. Microb. Infect., 13: 25-32.
5. Fisher, M.C., 2009. Endemic and introduced haplotypes of *Batrachochytrium dendrobatidis* in Japanese amphibians: sink or source? Mol. Ecol., 18: 4731-4733.
6. De Paula, C.D. and J.L. Catão-Dias, 2011. Chytridiomycosis: a Devastating emerging fungal disease of amphibians. Braz. J. Vet. Pathol., 4(3): 250-258.
7. Hof, C., M.B. Araujo, W. Jetz and C. Rahbek, 2011. Additive threats from pathogens, climate and land-use change for global amphibian diversity. Nature, 480: 516-519.
8. Stuart, S.N., M. Hoffmann, J.S. Chanson, N.A. Cox, R.J. Berridge, P. Ramani and B.E. Young, 2008. Threatened amphibians of the world. Barcelona, Spain: Lynx Edicions, in association with IUCN, Conservation International and Nature Serve.
9. Donnelly, M.A. and C. Guyer, 1994. Mark recapture. In Measuring and monitoring biological diversity, standard methods for amphibians, W.R. Heyer, M.A. Donnelly, R.W. McDiarmid, L.C. Hayek and M.S. Foster (eds.). Smithsonian Institution Press, Washington, D.C., pp: 183-205.

10. Eden, C.J., D.H. Whiteman, L. Gray and A. Wissinger, 2007. Accuracy assessment of skeletochronology in the Arizona tiger salamander (*Ambystoma tigrinum nebulosum*). Copeia, 2: 471-477.
11. Berger, B.L., R. Speare and A.D. Hyatt, 1999. Chytrid fungi and amphibian declines: overview, implications and future directions. In Declines and disappearances of Australian frogs, A. Campbell (ed.). Environment Australia, Canberra, Australia, pp: 23-33.
12. Olsen, V., A.D. Hyatt, D.G. Boyle and D.M. Endez, 2004. Co-localization of *Batrachochytrium dendrobatidis* and keratin for enhanced diagnosis of chytridiomycosis in frogs. Dis. Aquat. Organ., 61: 85-88.
13. Mazzoni, R., A.C. Cunningham, P. Daszak, A. Apolo, E. Per domo and G. Speranza, 2003. Emerging pathogen of wild amphibians in frogs (*Rana catesbiana*) farmed for international trade. Emerg. Infect. Dis., 9(8): 995-998.
14. Woodhams, D.C., R.A. Alford and G. Marantelli, 2003. Emerging disease of amphibians cured by elevated body temperature. Dis. Aquat. Organ., 55: 65-67.
15. Bosch, J., I. Martinez-Solano and M. Garcia-Paris, 2001. Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricians*) in protected areas of central Spain. Biol. Conserv., 97: 331-337.
16. Bradley, G.A., P.C. Rosen, M.J. Sredl, T.R. Jones and J.E. Longcore, 2002. Chytridiomycosis in native Arizona frogs. J. Wildl. Dis., 38: 206-212.
17. Lara, J. Rachowicz and V.T. Vredenburg, 2004. Transmission of *Batrachochytrium dendrobatidis* within and between amphibian life stages. Dis. Aquat. Organ., 61: 75-83.
18. Boyle, D.G., D.B. Boyle, V. Olsen, J.A.T. Morgan and A.D. Hyatt, 2004. Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real time Taqman PCR assay. Dis. Aquat. Organ., 60: 141-148.
19. Berger, L., A.D. Hyatt, V. Olsen, S.G. Hengstber, D. Boyle, G. Marantelli, K. Humpreys and J. Longocore, 2002. Production of polyclonal antibodies to *Batrachochytrium dendrobatidis* and their use in an immunoperoxidase test for chytridiomycosis in amphibians. Dis. Aquat. Organ., 48: 213-220.
20. Parto, P., S. Vaissi, H. Farasat and M. Sharifi, 2013. First report of Chytridiomycosis (*Batrachochytrium dendrobatidis*) in endangered *Neurergus microspilotus* (Caudata: Salamandridae) in Western Iran. Global Veterinaria, 11(5): 547-551.
21. Sharifi, M. and S. Assadian, 2004. Distribution and conservation status of *Neurergus microspilotus* (Caudata: Salamandridae) in western Iran. Asiatic Herpetol. Res., 10: 224-229.
22. Naderi, B., 2011. On ecology of *Neurergus microspilotus* and *N. Kaiseri*. A thesis for a MSc degree on Zoological Systematic, Dept of Biology, Faculty of Science, Razi University, Kermanshah, Iran.
23. Sharifi, M., H. Farasat and S. Vaissi, 2012. Sexual size dimorphism in *Neurergus kaiseri* (Caudata: Salamandridae) in southwestern Zagros Mountains, Iran. Amphibian and Reptile Conservation, 6: 1-8.
24. Nazan, Ü., 2009. A skeletochronological study of age, growth and longevity in a population of the *Caucasian Salamander*, *Mertensiella caucasica* (Waga, 1876) (Caudata: Salamandridae) from Turkey. J. Zool., 5(1): 74-84.
25. Khonsue, W., T. Chaiananporn and P. Pomchote, 2010. Skeletochronological assessment of age in the Himalayan crocodile newt, *Tylototriton verrucosus* (Anderson, 1871) from Thailand. Tropical Natural History, 10: 181-188.
26. Kutrup, B., E. Cakir, Z. Colak, U. Bulbul and H. Karaoglu, 2011. Age and growth of the green toad, *Bufo viridis* (Laurenti, 1786) from an island and a mainland population in Giresum, Turkey. J. Anim. Vet. Adv., 10(11): 1469-1472.
27. Brito-Gitirana, L.D., F.A. Felsemburgh, S.P.D.C. Silva and P.G.D. Almeida, 2009. Is the Chytrid fungus really responsible for amphibian decline? Open Zool. J., 2: 49-52.
28. Kerry, M.K. and J.M. Hero, 2006. Survivorship in wild frogs infected with chytridiomycosis. Eco. Health., doi: 10. 1007/s10393-006-0027-7.
29. Takesh, M., 2011. Ecological appraisal (distribution, population estimate, relative abundance, phenology) of the yellow spotted mountain newt Neurergus microspilotus. A thesis for a MSc degree on Zoological Systematic, Dept of Biology, Faculty of Science, Razi University, Kermanshah, Iran.

30. Carey, C., J.E. Bruzgul, L.J. Livo, M.L. Walling, K.A. Kuehl, B.F. Dixon, A.P. Pessier, R.A. Alford and K.B. Rogers, 2006. Experimental exposure of Boreal Toads (*Bufo boreas*) to a pathogenic chytrid fungus (*Batrachochytrium dendrobatidis*). Eco. Health., 3: 5-21.
31. Rachowicz, L.J. and J.C. Briggs, 2007. Quantifying the disease transmission function: effects of density on *Batrachochytrium dendrobatidis* transmission in the Mountain Yellow-legge Frog *Rana muscosa*. J. Anim. Ecol., 76: 711-721.
32. Walker, S.F., M.B. Salas, D. Jenkins, T.W.J. Garner, A.A. Cunningham, A.D. Hyatt, J. Bosch and M.C. Fisher, 2007. Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate. Dis. Aquat. Organ., 77: 105-112.
33. Baloutch, M. and H.G. Kami, 1995. Amphibians of Iran. Tehran University Press. Tehran. Iran, pp: 177. (In Persian).
34. Ron, S.R., 2005. Predicting the distribution of the amphibian pathogen *Batrachochytrium dendrobatidis* in the New World. Biotropica, 37(2): 209-221.
35. Piotrowski, J.S., S.L. Annis and J.E. Longcore, 2004. Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. Mycologia, 96(1): 9-15.
36. Symonds, E.P., H.B. Hines, P.S. Bird, J.M. Morton and P.C. Mills, 2007. Surveillance for *Batrachochytrium dendrobatidis* using Mixophyes (Anura: Myobatrachidae) larvae. J. Wildl. Dis., 42: 48-60.
37. Rayan, M.J., K.R. Lips and M.W. Eichholz, 2008. Decline and extirpation of an endangered Panamanian stream frog population (*Craugastor punctariolus*) due to an outbreak of chytridiomycosis. Biol. Conserv., 141: 1636-1647.
38. Hossack, B.R., M.J. Adams, E.H.C. Grant, C.A. Pearl, J.B. Bettaso, W.J. Barichivich, W.H. Lpwe, K. True, J.L. Ware and P.S. Corn, 2010. Low prevalence of Chytrid fungus (*Batrachochytrium dendrobatidis*) in amphibians of U.S. headwater streams. J. Herpetol., 44: 253-260.