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Survey on fungal, parasites and epibionts infestation on the *Astacus leptodactylus* (Eschscholtz, 1823), in Aras Reservoir West Azarbaijan, Iran

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Abstract

A total of 394 (255 males,139 females) live freshwater crayfish *Astacus leptodactylus* from four stations of Aras reservoir in West Azarbaijan Province (North-Western Iran) were studied during the winter until early autumn of 2009 for the presence of parasites, Epibionts and Fungal agents. Parasitological surveys were carried out on gills; exoskeleton and internal organs, mycological examinations on the exoskeleton (the legs, abdominal cuticle and the eggs). 9 epibionts and parasites peritrich protozoans including :*Cothurnia sieboldii* (68.5%), *Zoothamnium* spp. (56.6%), *Vorticella similis* (45.6%), *Chilodonella* spp. (0.5%), *Podophrya fixa* (7.8%), *Epistylis chrysemidis* (53.2%), *Pyxicola annulata* (66%), *Opercularia articulata* (19.8%), *Tetrahymena pyriformis* (0.5%) were recorded. From Metazoan parasites group, *Branchiobdella kozarovi* (71%) as the first observation was the only parasite recorded from exoskeleton with prevalence (100%) during spring and summer of the study year. Infected gills were heavily damaged with *Aeolosoma hemprichi* (Annelid) in winter with 90% prevalence. Other Epibiont fouling organisms such as Rotatoria; free living Nematods were observed in this survey. Furthermore, on the mycotic agents identified *Penicillium expansum*; *Aspergillus flavus; Alternaria* sp. ; *Fusarium* sp. and *Saprolegnia* sp. were isolated in IM media and identified with slides cultured from cuticular melanized lesions and eggs of infected specimens. This is the first investigation on epibionts, parasites and fungal organisms of the endemic crayfish in Aras reservoir, Iran.

Keywords: *Astacus leptodactylus*, Fungi,Epibiont, Aras reservoir, Iran

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Introduction

Aras dam is one of the important water sources for irrigation and electric power in Iran which was constructed on Aras River and completed in 1970 with a total area of about 11000-15000 (ha). Fish composition species in Aras Lake included 15 species belonging to Cyprinidae 86%, Siloridae and Percidae individualy 7% (Azadikhah et al., 2008). Furthermore, *Astacus leptodactylus* is the most economic non-fish species introduced in Aras reservoir. Fresh water crayfish *Astacus leptodactylus* with a limited geographical distribution in the country is the only crayfish existing in Iran. This species is in Anzali lagoon where it is translocated to a few rivers and reservoirs in the Northwestern part of the country. The amount of the local consumption of crayfish in Iran is very low (Gorabi, 2003). Therefore, almost all of the catches are exported to European countries (shilat, 2009). Pathological investigations are essential either obtaining information about the health status of wild populations or guaranteeing the success of crayfish culturing by preventing the spread of diseases. The two most dangerous pathogenic groups are fungal and viral. Of these, the most virulent toward crayfish is the fungus, *Aphanomyces astaci*, and the agent of crayfish plague in freshwater crayfish (Edgerton et al., 2002a). Some authors have reported the association of an intranuclear bacilliform virus with the near extirpation of *Astacus pallipes*, complex from the Nant watershed in France (Edgerton et al., 2002a; Edgerton, 2003; Edgerton et al., 2004). A serious mortality in crayfish populations has often been attributed to pollution but without any proof. However, the causative agent in many cases of mass mortality remains undiagnosed (Edgerton et al., 2004). Except Asgharnia (2008) who described prevalence and intensity of *Branchiobdella hexodenta* (Annelid: Clitella) in carapace and gills of cultured *Astacus leptodactylus* in research ponds in the north of Iran. This study completely focused on fungal, epibionts and parasites on cultured *Astacus leptodactylus*'s natural population in Iran. But among the neighboring countries, the epibiont and parasitic infections have been well studied in Turkey. The parasite fauna of crayfish was investigated by Harioglu (1999), some studies carried out by Baran et al. (1987) and Baran and Soylu (1989) on the parasite fauna of cultured and wild crayfishes of Turkey may concern similar problems as those of the neighboring Northwestern part of Iran where Aras basin is situated. Concerning mycotic disease (plague) of crayfish, which is a well-known infection to Europe and especially in Turkey, research work done by Söderhäll, K., and Cerenius, L. (1999) confirmed that *Astacus leptodactylus* is the most sensitive species among other members of Astacus. From 1985, the European Union and Turkey are well known countries where the plague has been established. The objective of the study was to evaluate the health status of native freshwater crayfish population in North-West of Iran.

Material and methods

During the winter until early autumn of 2009 (Jan-October), a total of 394 (255 males, 139 females) live fresh water crayfish *Astacus leptodactylus*, from wild populations were collected from Aras

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reservoir in the region of N-W Iran. The samples were collected from four different stations (Figure 1): 1. 2. 3. 4. Specimens were captured by means of baited traps. Only live crayfish were rapidly transported individually by foam bags and were maintained in + 4°C till 24 hours until being examined in the Poldasht laboratory (Table I). In the laboratory, specimens were first subjected with bioexamination and then parasitological examinations were carried out.

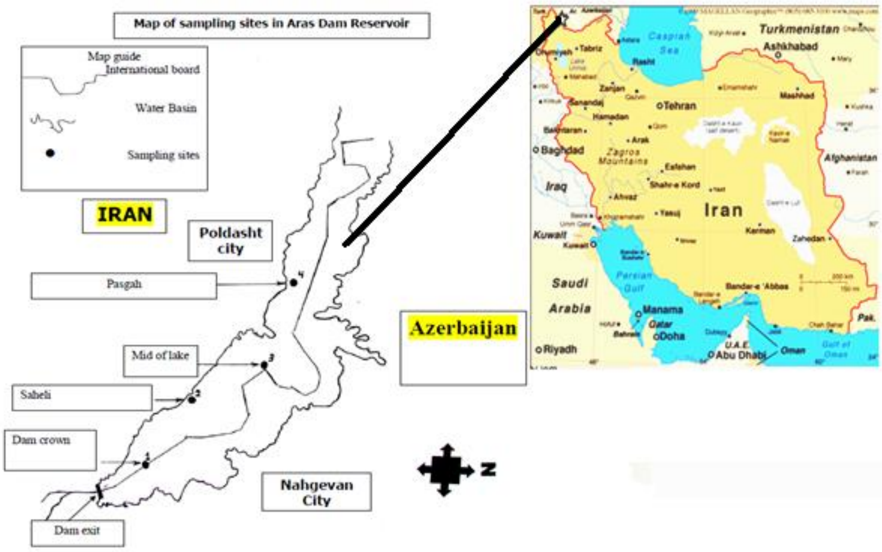

Figure 1: Location of sampling stations on Aras reservoir; West Azarbaijan; Iran

Parasitological investigation

Preparations of wet smears from the exoskeleton, lesions and gills of each specimen were as follows: the mucus was scraped separately from different organs on to a micro slide and then it spread the mucus carefully with the cover slide. The protozoas exposed to a fixative for at least 15 minutes and then washed for several minutes in alcohol containing a drop of added iodine solution. Then, both wet and dry smears were mounted in Canadabalsam often dehydration in accordance with Fernando et al. (1972). Gills need special attention due to the sensitivity of this organ to epibiont, parasites and its important rule in osmoregulation. The organ was cut off and examined under a microscope at X100-400 magnification. Metazoan parasites were separated as the same methods used for external epibionts. Line drawing and length of the species were measured by computer and projected by a video camera. Measurements of the parasites were related to the scale of an objective micrometer, projected to the screen in the same way. The validity of the methods was checked by measuring the same organs with microscope micrometers. For the identification of epibionts and parasites the keys given by Hoffman (1967); Matthes and Guhl (1973); Kudo (1977) and Alderman et al. (1988) were used. Branchiobdellidans, were removed from

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each specimen, fixed in 70% ethanol and were then counted and clarified with lactophenol. The specimen were stained with Borax Carmine and mounted with Glycerin Jelly or Hoyer's fluid. All of the branchiobdellidans were examined and measured using the optical microscope. Identification of the specimens was made on the basis of the jaws, the spermatheca and spermathecal duct morphology using identification keys (Moszynsky, 1938; Pop, 1965; Gelder et al., 1994).

Mycological investigation

Small pieces (1-2 mm^2) and melanized patches of the exoskeleton were removed from the abdomen and legs of infected specimens among 390 crayfishes, rinsed in sterile distilled water, placed on plates containing glucose-yeast extract agar (Min et al., 1994) with penicillin G (6 mg l^{-1}) and oxolinic acid (10 mg l^{-1}) (Alderman and Polglas, 1986), and incubated at 26°C. The colonies found growing on agar were examined macro- and microscopically, using bright field illumination and slide culture method, for identification. Moulds were identified to genus using morphological features, i.e. colony appearance, hyphae, sexual and/or asexual reproduction (Barron, 1968; St–Germain and Summerbell, 1996; De Hoog and Guarro, 1996). When the colonies had tubular, variably branched, very poorly septate, hyphae (looking like oomycetes), they were placed in sterile distilled water with at 18°C and 26°C, to develop sporangia and/or sexual structures.

Table 1: Number of captured and type of examination crayfishes during winter until early autumn (2009)

Sampling stations	Captured Crayfish		Total	Type of Examination							
				Parasitological				Mycological			
	Meal	Female		Win	Spr	Sum	Aut	Win	Spr	Sum	Aut
1	66	48	114	25	25	27	37	16	4	2	6
2	61	36	97	24	27	26	20	11	5	3	2
3	63	28	91	24	25	23	19	10	4	1	3
4	65	27	92	24	24	23	21	7	6	3	2
Total	255	139	394	97	101	99	97	42	19	9	13
	394			394				83			

*Win=Winter , Spr=Spring , Sum=Summer , Aut=Autumn

Results

Epibiont investigation

The parasite and epibiont organisms which were removed from the examined *Astacus leptodactylus* are summarized in Table 2. Peritrichous ciliate *Cothurnia sieboldii*, *Epistylis* sp., *Vorticella similis*, *Zoothamnium* sp., *Pyxicola annulata*, *Opercularia articulata* and *Podophrya fixa* were found on crayfish from all stations, while *Chilodonella* sp. and *Tetrahymena pyriformis* showed a lower infestation rate only in one station (Figure 2). In laboratory examination externally, the most frequently observed organisms were *Cothurnia sieboldii*, on healthy

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exoskeleton. Colonies of these ciliates tended to concentrate in protected cuticular folds on the exoskeleton and cephalothorax (Figure 3), and in the gill chamber particularly in well-sheltered areas, such as at the base of gill filaments. When peritrichous ciliates were located in the deeper portions of the gill chamber, they appeared to trap debris and bacteria. Besides the other epibiont protozoan including: *Stylonychia mytilus*, *Paramecium* sp., are observed with low incidence one the branchial cavity of crayfish.

Table 2: Results of parasitological examination – No. of positives (%)

Sampling Stations	1	2	3	4	Infected organ
No. Crayfish Examined	114	97	91	92	-----
Aeolosoma hemprichi	60(53.1)	53(54.6)	45(49.5)	47(51.1)	G
Cothurnia sieboldii	60(52.6)	53(54.6)	45(49.5)	47(51.1)	G
Podophrya fixa	15(13.2)	9(9.3)	3(3.3)	4(4.3)	C
Epistylis sp.	52(45.6)	61(62.9)	48(52.7)	45(48.9)	Pl, G
Vorticella similis	53(46.5)	48(49.5)	40(44%)	39(42.4)	G, L
Zoothamnium sp.	61(53.5)	56(57.7)	55(60.4)	51(55.4)	C
Chilodonella sp.	2(1.8)	0(0)	0(0)	0(0)	G
Fresh water Nematodes	50(43.9)	52(53.6)	49(53.8)	48(52.2)	Pl, G
Cocoons of *B. kozarovi*	17(14.9)	15(15.5)	18(19.8)	14(14.1)	Pl, C
Branchiobdella kozarovi	83(72.8)	70(72.2)	64(70.3)	63(68.5%)	G,E, C, A ,R , Pl
Philodina acuticornis	17(14.9)	15(15.5)	18(19.5)	13(14.1)	G
Opercularia articulata	32(28)	21(21.6)	11(12)	14(15.2)	G, Pl
Pyxicola annulata	65(65.8)	68(70.1)	62(68.1)	65(70.6)	G
Tetrahymena pyriformis	2(2)	0(0)	0(0)	0(0)	G, M

G =gill, Pl= pleopod, L=legs=cephalothorax, E=eyes, A=antenna, R=rostrum, M= muscle

Branchiobdella kozarovi was found and identified on the exoskeleton and walking leg, antenna; antennules; base of eyes and in one of the specimens of the gill of crayfishes from all of the stations. The most infestation was observed on 3rd Maxilipede (Figure 3). The percentage of infestation of *B.kozarovi* isolated in crayfishes is showed in Table II. The mean total length of specimens was 1.35±0.6 mm (n=30, range 0.8-3 mm,) with a mean width of 0.32±0.09 mm (n=30, range 0.22-0.6 mm).

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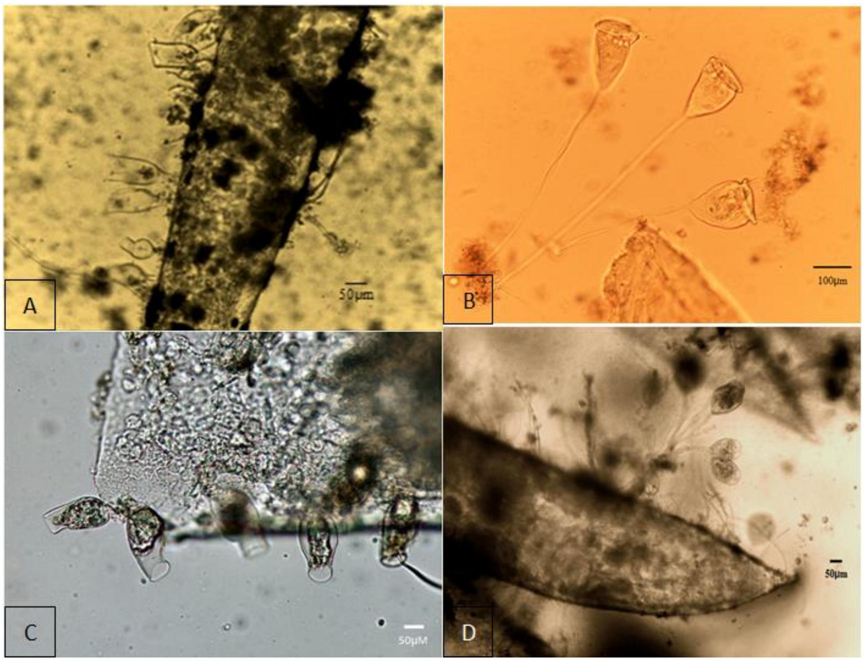

Figure 2: A=Loricate ciliophora *Cothurnia sieboldii* (100X), B=*Vorticella similis* (400X), C= *Pyxicola annulata* (400X)., *D= Opercularia articulate* (100X), Attached to the gill filaments of *Astacus leptodactylus*

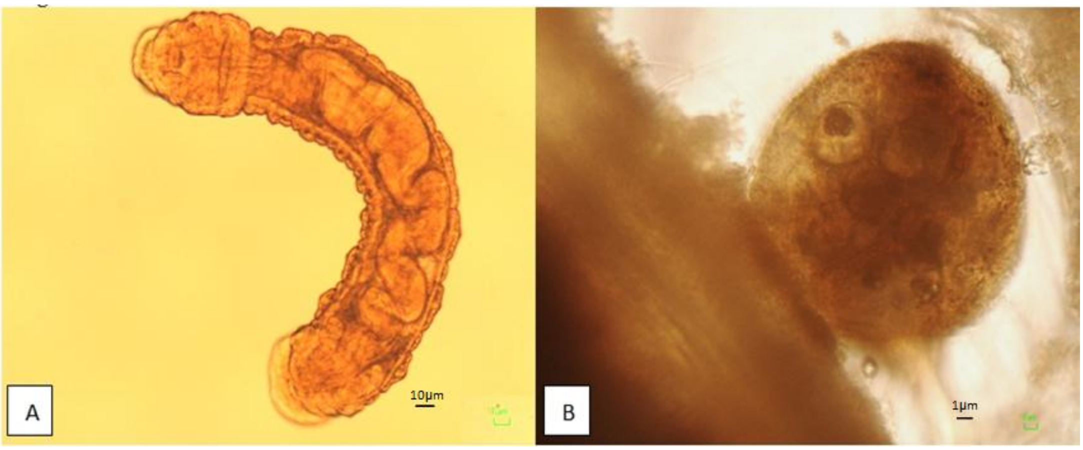

Figure 3: A=3rd Maxilipede infestation by *Branchiobdella kozarovi* (100X), B=Peleopods infestation by cocoons of *Branchiobdella kozarovi* (1000X)

The numerous cocoons were attached on the thorax segments (exopodit, protopodit, epipodit) of crayfishes (Figure 3). High infestation in the presence of branchiobdellidan and their cocoons were observed in the exoskeletons of samples during spring and summer (100%). Metazoan ectosymbionts included: *Aeolosoma hemprichi* (Annelida), *Philodina acuticornis* (Rotatoria), *Mesocyclops strennus* of copepods, and free living nematodes including: *Mononchus* sp. ,*Prodesmodora* sp. and *Bunonema reticulatum* are observed on gill chambers of examined specimens in all seasons.Fungi were isolated from both the abdominal cuticle and the legs, and the results are summarized in Table 3. *Penicillium expansum*; *Aspergillus flavus; Alternaria* sp. and *Saprolegnia* sp. were isolated from 83 captured out of 394 specimens after cultured in IM media from cuticular melanized lesions and eggs. *Penicillium expansum* was the most frequently isolated being found on 16.8% of the abdomens melanized cuticles, 15.7% on legs and 16.1% on eggs of examined specimens. Most of these crayfishes were with marked clinical signs. Seasonal prevalence of mycoting infection was as follows: Winter (53%), Spring (14.4%), Summer (3.6%) and Autumn (10.8%) in the study year. Positive septate hyphae on the exoskeleton were often observed in the presence of melanized areas and in association with detritus and bacteria accumulations. A mycete with coenocytic thallus from the melanized areas of the exoskeleton and legs on one crayfish was isolated. This, when transplanted on to IM media, produced abundant clavate, pyriform or irregular gemmae, single, or frequently, catenulate. Cylindrical, clavate or irregular straight zoosporangia were abundant. The strain failed to produce a sexual form at either 18°C or 26°C. The characteristics of secondary cysts were not observed. Based on morphological features, the isolates were assigned to the genus *Saprolegnia*, but further identification should be carried out to the species level.

Table 3: Results of mycological investigations: no. of positives (percent).

Seasons		Winter	Spring	Summer	Autumn	Total
No. of crayfish examined		42	19	9	13	83
Legs (walking pleopod)	*Penicillium expansum*	8(19)	2(10.5)	1(11.1)	2(15.4)	13(15.7)
	Aspergillus flavus	5 (11.9)	1(5.2)	1(11.1)	3(23)	10(12)
	Fusarium sp.	2 (4.7)	0	0	0	2 (2.4)
	Saprolegnia sp.	1(2.4)	0	0	0	1(1.2)
	Negative	26(61.9)	16(84.3)	7(77.7)	8(61.5)	57(69.3)
Abdominal cuticle	*Penicillium* expansum	9(21.4)	3(15.7)	1(11.1)	1(7.7)	14(16.8)
	Aspergillus flavus	3(7.1)	1(5.3)	0	1(7.7)	5(6)
	Alternaria sp.	0	1(5.3)	0	1(7.7)	2(2.4)
	Saprolegnia sp.	1(2.4)	0	0	1(7.7)	2(2.4)
	Negative	29(69)	15(78.9)	8(88.8)	9(69.2)	12(14.4)
Eggs	*Penicillium expansum*	3(25)	2(10.5)	0	0	5(16.1)
	Aspergillus flavus	2(16.6)	2(10.5)	0	0	4(12.9)
	Saprolegnia sp.	10(83.3)	3(15.8)	0	0	13(41.9)
	Negative	27(64.2)	12(63.1)	0	0	39(47)

Discussion

A variety of protozoa generally move about or attach to the body surface and gills of crayfish (Johnson, 1983). Epibiont infestation of the gills and exoskeleton of the crayfish examined in our study were common, but no detrimental effects on crayfish health were macroscopically observed. In another study, carried out on red swamp crayfish (*Procambarus clarkii*), Quagliof et al. (2004) found that the elevated presence of *Epistylis* spp. was associated with low dissolved oxygen concentration and high organic pollution. The low incidence of *Epistylis* spp. in examined *Astacus pallipes* complex could be related to the optimal environmental conditions and water quality which they experience. Matthes and Guhel (1973) have reported *Cothurnia sieboldii* also as a commonly occurring species on European crayfish. Peritrich infestations of freshwater crayfish have been widely reported (Edgerton et al., 2002b). In our survey, sessile peritrichs are found on the external surfaces, including the branchial chamber. Different species of peritrich ciliates show site specificity, some being found predominantly on the gills, some on the appendages and carapace, others distributed widely over most of the body (Table 2). Among the epibiont and parasitic population found, most attention should be paid to *Branchiobdella kozarovi* which may intensify the success of production in future development plans. In North America, Europe and East Asia a wide range of crayfish species are infected by *Branchiobdella kozarovi*. This report is the first related to parasite and epibiont organisms and *Branchiobdella kozarovi* infestation on *Astacus leptodactylus* in Iran. On the basics of our finding the mycetes of the genera *Penicillium expansum*, *Alternaria* spp., *Fusarium* spp. and *Aspergillus flavus* were found in healthy specimens. They can be considered naturally occurring saprophytes, often associated with poor water quality. Chinain and Vey (1988) reported disease caused by *Fusarium solani* in the crayfish species *Astacus leptodactylus* and *Pacifastacus leniusculus* in Europe. They found that of the two species of freshwater crayfish that were studied, *Astacus leptodactylus* was the more susceptible species. Other species of fungi which have been reported as epizoites of freshwater crayfish include *Alternaria spp., Hormodendrum spp., Aspergillus spp., Saprolegnia spp., Uncinula spp. and Hormisum spp*. which were found on the external surfaces of Northern American crayfish species, *Pacifastacus simulans*, *Pacifastacus clarkii*, *Pacastacus zonangulus* and *Fallicambarus hedgpethi* (Lahser, 1975). *Saprolegnia spp*. is common water mould and includes species which are responsible for significant infections involving both living and dead fish. In the strain of *Saprolegnia spp*. Only the form of asexual reproduction was isolated. Due to lack of the formation and observation in the characteristic of secondary cysts and the molecular studies we were couldn’t to identify the species. As pointed out by Söderhäll et al. (1991), although *Saprolegnia parasitica* causes a severe problem in fish, it does not appear to be an important parasite for crayfish. The populations of *Astacus leptodactylus* sampled of Aras reservoir during the survey is rather in good favorable environmental conditions and habitat. Opportunistic pathogens and epibionts were frequently observed in examined specimens in the wild and in captivity. Therefore, particular care must be given to crayfish culture to prevent environmental stressors causing disease outbreaks. The presence of the native crayfish has been greatly reduced progressively in number, in the recent years in many reservoirs of Iran. Thus, this species has risk extinction. This may be the result of epizootic diseases, the introduction of alien species, changes in the habitat brought by excavation; work on river-beds and streams and general industrialization which very often occur in areas close the course of the rivers. In general the epibionts, peritrichous protozoan and metazoan parasites of *Astacus leptodactylus* are common fauna of fresh water crayfish. Most of them attach to the exoskeleton and gills of crayfish, and feed primarily on bacterial cells associated with eurotropic reservoir which generally increase in summer and reduce in winter. Therefore, we can come to a conclusion that parallel to increasing of eutrophication of Aras reservoir, prevalence and intensity of epicommensals like *Epistylis* spp. on crayfish population will be significantly increased and may have an adverse effect on health status which may lead to disease outbreak and mortality.

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