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Parasitism (*Flamingolepis liguloides* Gervais, 1847) with High Prevalence in Brine Shrimp Population from Çamaltı Saltworks

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ABSTRACT
Populations of the species *Artemia* (Brine shrimp) in saltworks have become a popularity popular field of study for aquaculture for its significance for aquatic ecosystems as being an important source of sustenance for water birds in hypersaline food webs. Besides, species in the genus *Artemia* are the intermediate host of severe cestode species which are associated with flamingos. This study reports on the prevalence of native *Artemia parthenogenetica* parasitism of *Flamingolepis liguloides* in the Çamaltı saltern ecosystem in the Gediz wetland between January and December 2022 in Türkiye. Infected *A. parthenogenetica* was sampled from April to September 2022 in salt pans where flamingo birds and salt production are available for parasitological diagnosis. The parasites were determined in the abdomen, thorax and near the gut tract of *A. parthenogenetica*. The highest prevalence of parasite was found in juvenile individuals as 86.67±1.45% and adults as 76.06±1.16% in May. The main intensity was varied from 1.18±0.01 to 1.92±0.06 parasites per infected host depending on the sampling months and age of the brine shrimp. The most abundant parasite infestation was recorded as 1.44±0.02 parasites per investigated host in juvenile brine shrimp in June since seasonal conditions are favorable for such infestation.

Keywords: Aquaculture, Brine shrimp, Avian, Parasitism, Solar saltworks, Wetlands, İzmir, Türkiye

1. Introduction

The cosmopolitan brine shrimp *Artemia* (Branchiopoda, Anostraca) is one of the most studied aquatic organisms based on its broad use in ecotoxicology, ecology, developmental and evolutionary biology, feeding of aquatic organisms and the extensive use in the aquaculture industry (Büke 2002; Kırkağaç et al. 2017; Kaska 2019). Very saline waters, i.e. hypersaline waters, are unique extreme habitats where salinity limits species richness. There are major patterns of relationship between salinity and species richness of free-living aquatic animals in such ecosystems, but general regularities for parasitic organisms have yet to be established. All 85 species and forms of parasites found in hypersaline waters belong to five phylum: Platyhelminthes, Nematoda, Acanthocephala, Cnidaria and Arthropoda. Platyhelminthes is the most diverse and species-rich branch of the Cestoda class. Most species are found in hypersaline waters with salinities not exceeding 100 g/L. The total number of parasite species decreases exponentially with increasing salinity due to cellular osmotic stress in the organism. For this reason, the number of free living animal species living in waters with salinities between 35 and 210 g/L is approximately 12 times higher than that of parasites in all ranges of this salinity range. Salinity affects parasite richness and composition in two ways: directly and through the availability of hosts. Free-living crustaceans are the suitable hosts of most parasite species in hypersaline waters. The *Artemia* species, the most halotolerant, is a good intermediate host for 22 species and unidentified parasite forms (Kornyychuk 2023). The *Artemia* species are reported as the keystone taxon in hypersaline food webs with different status; they are the main prey of aquatic birds, the intermediate host for several parasite species and the primary consumer of phytoplankton in the ecosystem (Georgiev et al. 2005; Sánchez et al. 2006; Vasileva et al. 2009; Rode et al. 2013 a,b). Specifically, brine shrimps are stated as the main prey of breeding flamingos (Britton & Johnson 1987; Bechet & Johnson 2008) and then brine shrimps are also reported as intermediate hosts of parasite cestodes.

In the scientific record, the first description of a cysticercoid in *Artemia* species was reported approximately 100 years ago (Heltdt 1929). There are now 22 cestode species from the genus *Artemia* (Redón et al. 2020). Five of these species belong to the genus *Flamingolepis* (Cyclophyllidea, Hymenolepididae) (Maksimova 1979) with *F. liguloides* (Gervais 1847) being the most common (Amarouayache et al. 2009). The first invasion of *Artemia* sp by larval forms of cestoda was reported from Tunisia (Heltdt 1929), then many other countries, such as Spain (Amat et al. 1991a,b; Sánchez et al. 2013), France (Gabrion & MacDonald 1980; Thiery et al. 1990), Italy (Mura 1995) and Türkiye (Koru 2022). The parasites spread to the birds with the trophic transmission when feeding on infected *Artemia* species (Sánchez et al. 2013). It then develops into mature worms in the digestive tract of birds and the eggs of the adult parasite spread around the environment via the faeces of the main host (Amarouayache et al. 2009).

In this study, after the detection in 2018 (Koru 2022), the spread of the *Flamingolepis liguloides* (Gervais 1847) parasite in *A. parthenogenetica* (Barigozzi 1974) in the Çamaltı saltern ecosystem was investigated. The study aims to determine the temporal dynamics of bird parasites by means of *A. parthenogenetica* in the hypersaline saltworks ecological system, which is of great importance for wildlife. The area is also the breeding ground of flamingos and the results of this study reveal the presence of this parasitism in the Eastern Mediterranean.

2. Material and Methods

2.1. Study area

The study was performed in Çamaltı Saltern (Bird sanctuary biological area of İzmir), which is the largest sea-sourced saltworks in Türkiye, established in the Gediz River basin (Gediz wetland), 28 km away from Izmir. The size of the study area is 60 square kilometers, with a water depth of 1- 4 m and located at 38º 30´ 18″ N, 26º 54´ 55″ E district. The connection of the saltpan to the Aegean Sea is provided by pumps installed on the main channel. Thus, sea water can be gradually distributed to the sea salt crystallization pools and the wider area. Since the production and salt formation was finished in every October, the mechanical system that provides the water circulation was stopped. For this reason, there was no sea water inlet and outlet in the saltpans during the off season.

2.2. Sample collection and laboratory examinations

Brine shrimp (*A. parthenogenetica*) samples were collected monthly using a plankton net (125 µm mesh size) from three different salt pans of the Çamaltı Saltworks (38°30´12.73" N, 26°54´12.94" E) (Figure 1) from January to December 2022. The salinity parameters of the saltpans were measured using a refractometer (ATAGO Master-S28M, Tokyo, Japan), while the water temperature, oxygen and pH levels were measured using a multi-parameter probe (YSI ProQuatro, Ohio, USA) and recorded monthly during every sampling time at each sampling salt pans.

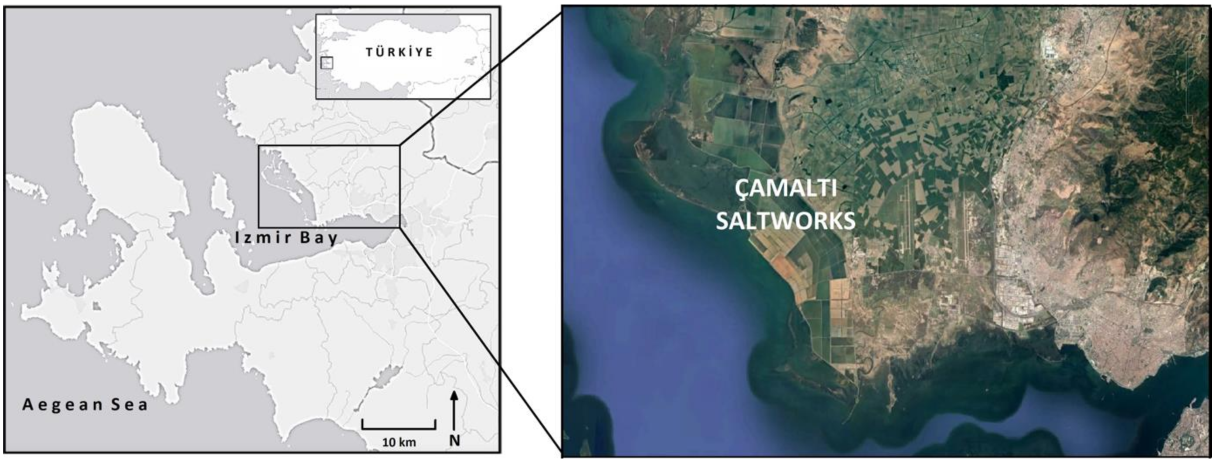

Figure 1- Geographical situation of the study area at Çamaltı saltern

The identification of the brine shrimps was carried out according to Brawne et al. (1991). The collected brine shrimp samples were washed with sterile physiological saline water and directly fixed in 3% formalin and preserved in 70% ethanol. The samples were mounted in temporary glycerol mounts and the mounted slides were examined under a light microscope (Olympus CX22RFS1) and parasites in the individual brine shrimp were counted. For each age group, 50 adults and 50 juvenile specimens were studied from each sampling station for every sampling month (a total of 150 brine shrimp specimens were studied for each age groups in every sampling months). Species identification of *F. liguloides* were performed according to Georgiev et al. (2005) and Redón et al. (2015) with oval cyst, elongated rostellar hooks and sucker hooklets, oval or round suckers. The prevalence, intensity and abundance of infection by month and host stages were calculated as follows (Bush et al. 1997):

$$Prevalence\ (\%) = \frac{Number\ of\ infected\ hosts}{Number\ of\ examined\ hosts} x100 \qquad (1)$$

$$Intensity\ (Number\ of\ parasites\ per\ infected\ host) = \frac{Total\ number\ of\ parasites\ recorved}{Total\ number\ of\ examined\ infected\ hosts} \tag{2}$$

$$Abudance\ (Number\ of\ parasites\ per\ examined\ host) = \frac{Total\ number\ of\ parasites\ recorved}{Total\ number\ of\ examined\ hosts} \tag{3}$$

2.3. Statistical analysis

A comparison of the prevalence, intensity and abundance in juvenile and adult brine shrimp were performed using one-way ANOVA, followed by Duncan's multiple comparison post-hoc test to determine the significance of mean prevalence, intensity and abundance between age groups in monthly. Investigations in the study were repeated three times. An alpha level of 0.05 was used to determine the significance ($P<0.05$). Data are expressed as means ± standard error (SE). The statistical analysis was performed using SPSS (version 23.0) software.

3. Results

The physicochemical and biological characteristics of wetlands may differ depending on the seasonal climatic conditions of that year. These differences also affect the characteristics of the entire life cycle in the ecosystem. Some important water parameters, such as pH, oxygen, and water temperature and salinity were recorded monthly in the salt pans where brine shrimp specimens were collected. These can be seen in Figure 2. During the study, pH, oxygen, temperature, and salinity values varied between 6.30±0.17-9.30±0.06, 3.90±0.06-8.07±0.07 ppm, 3.93±0.07-34.33±0.33 °C and 30.00±1.15-265.00±2.89%, respectively. The highest water temperature was observed in July and the highest salinity in September. The salinity changed significantly in the off season due to seasonal rainfall, and the production time of salt changed due to the water pumping from the sea to salt pans as well as evaporation. This made it impossible to collect brine shrimps throughout the entire year.

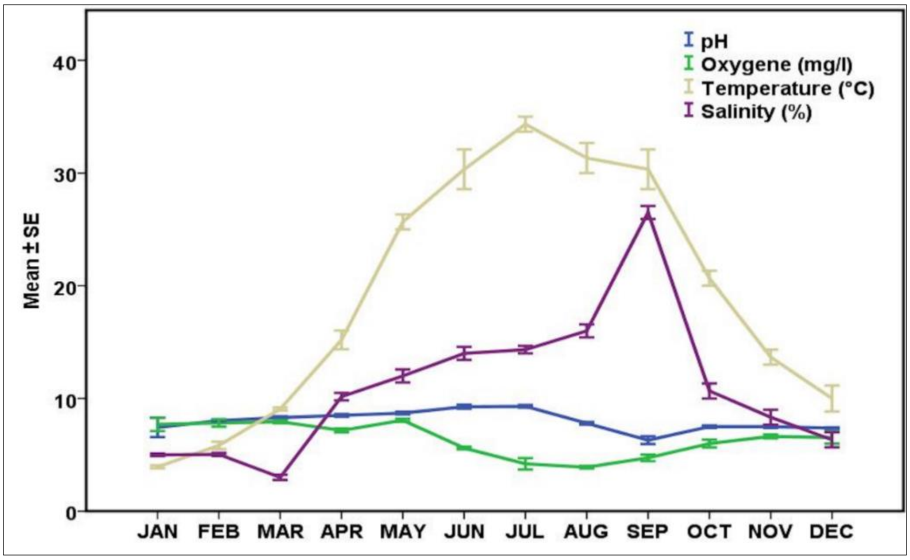

Figure 2- Detected annual water parameters from saltern area

The parasites were found in brine shrimp for five months, probably depending on abundance of great flamingos in wetland, the climate conditions and water regime. According to information received from ornithology experts, the number of migratory flamingo birds that came to the water in 2022 increased by $5x10^3$ individuals in comparison to the previous year of 2021. This means an increase in the transmission rate of the parasite through migratory flamingos, according to the results in the literature reports (Amarouayache et al. 2009). This causes a high population in flamingos due to the suitability of positive climate conditions and due to the suitable for biological conditions in the water. It has been determined that this causes an increase in ecosystem compared to previous years. *F. liguloides* infection was recorded from both juvenile and adult *A. parthenogenetica* individuals between May-September 2022. Brine shrimps were sampled from January to December 2022. The first infectation was detected in May in both juveniles and adults' examples. The main prevalence was found to be higher in juveniles than adult brine shrimps (df=9, F=494.35 p=0,00) ($P<0.05$) in May and June. The monthly prevalence of infestation is presented in Figure 3. The highest prevalence was detected as 86.67±1.45 % for juveniles and 76.06±1.16 % for adult brine shrimp specimens in May. A maximum of 7 and 4 cysticercoids were determined in infected adult and juvenile individuals, respectively. The mean intensity varied from 1.27±0.01 to 1.73±0.01 parasites per infected host in juveniles and from 1.18±0.01 to 1.92±0.06 parasites per infected host in adult brine shrimp. While the highest intensity value of juveniles was observed as 1.73±0.01 parasite per infected host in August, the highest intensity of adult brine shrimp was detected as 1.92±0.06 parasite per infected host in September. Differences in the intensity values of juveniles and adults are presented in Figure 4 (df=9, F=35.1, P=0,00). The mean abundance ranged between 0.82±0.02 and 1.44±0.02 parasite per investigated host in juveniles, 0.90±0.01 and 1.24±0.03

parasite per investigated host in adult brine shrimp. While the highest abundance of juveniles was detected in June, the highest abundance of adult brine shrimps was calculated in July. Differences between the mean abundance values of juveniles and adults were found to be statistically important (P<0.05) in all parasitism observed months (df=9, F=305.32 p=0,00) (Figure 5).

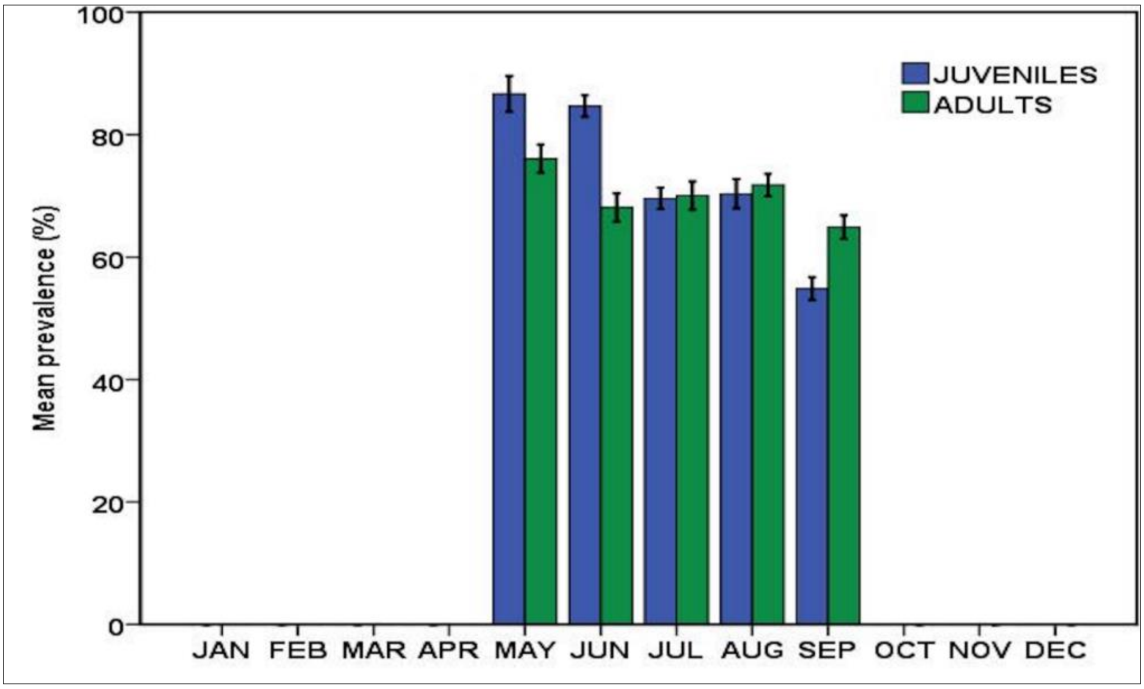

Figure 3- Monthly mean prevalence in juvenile and adult brine shrimps. (JAN, FEB, MAR, OCT, NOV, DEC) brine shrimp samples were not available. (MAY, JUN, JUL, AUG, SEP) in which the parasite was detected. Different letters indicate statistically differences at P<0.05 and error bars represented.

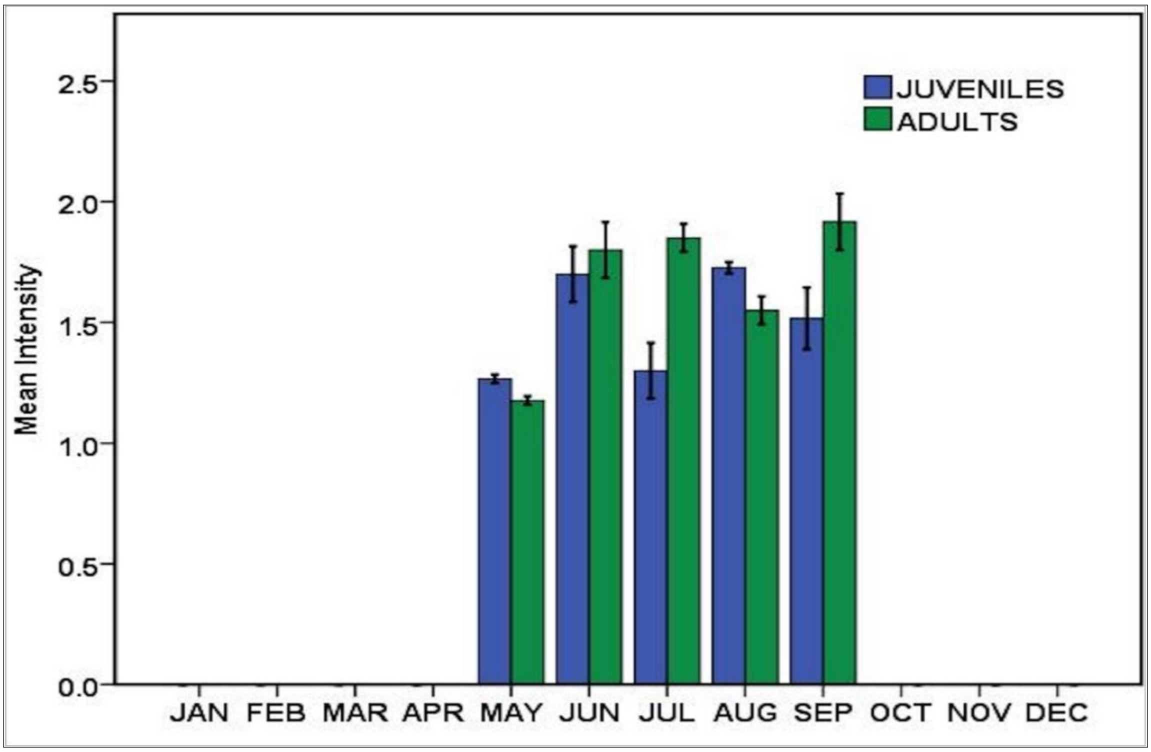

Figure 4– Monthly mean intensity in juvenile and adult brine shrimps. Egg&nauplii stage (JAN, FEB, MAR) brine shrimp samples were not available because of the end times of salt production. (OCT, NOV, DEC) parasitism was not detected (Salt production ponds were emptied because salt production ended. For this reason, there are no living things in the ecosystem). Different letters indicate statistically differences at P<0.05 and error bars represented SE.

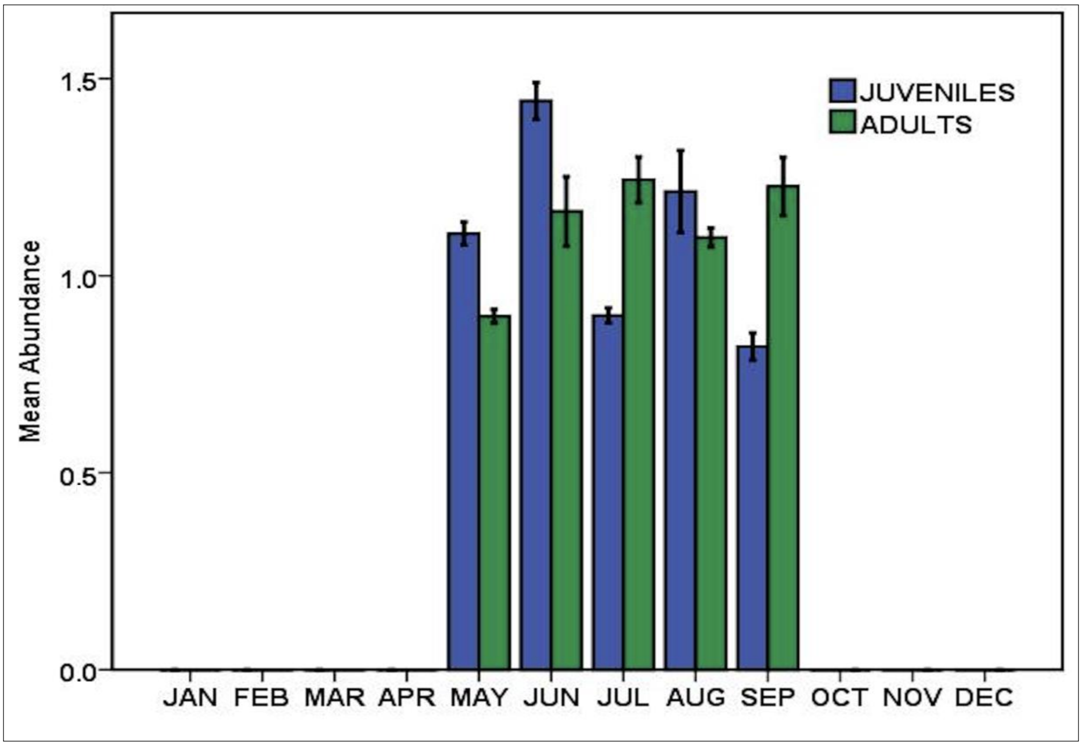

Figure 5– Monthly parasitism abundance in Çamaltı salt pans Brine shrimp samples were not available (JAN, FEB, MAR). Egg&nauplii stage; Monthly mean abundance in juvenile and adult brine shrimps (MAY, JUN, JUL, AUG). Parasitism was not detected (OCT, NOV, DEC). Different letters indicate statistically differences at P< 0.05 and error bars represented SE.

4. Discussion

The first reported parasitic infection of this species in *Artemia* from the Mediterranean region was recorded by Robert & Gabrion (1991). This study was carried out to determine the new status of the parasites of the *Artemia* species in the Çamaltı Region, Türkiye's largest sea salt ecosystem, compared to previous years. (Koru 2013; 2022). Field data were evaluated to determine the prevalence and seasonal effects of *F. liguloides*, which infects the *A parthenogenetica* host brine shrimp. The brine shrimp *Artemia* has been reported to be a non-selective filter feeder (Sánchez et al. 2013). Thus, the *Artemia* species have been feeding on all kinds of microorganisms, including detritus in the water column (Sánchez et al. 2007; Savage & Knott 1998). The *Artemia* species engulf the cestode eggs, which are called oncosphere (20 µm) and become cysticercoid (larva with scolex), into the hemocoel (Amarouayache et al. 2009; Robert & Gabrion 1991; Sánchez et al. 2007). The parasitism of *Artemia* sp. could be related to the existence of flamingos and relationship with the ecosystem. Therefore, the *F. liguloides* parasitism period is varied according the location of habitats. It was reported in *A. salina* from a wetland from Northeast Algeria at the end of winter (February/March), whereas the population of a second wetland area in Algeria was infected in spring (April/May) (Amarouayache et al. 2009). The parasite infection of *A. parthenogenetica* in Türkiye, was first recorded by Koru in 2022 (2022). This variation in the infection period was associated with the increase in the flamingo population density and the migratory behaviour of flamingos in wetland areas. Similarly, the parasitism of *F. liguloides* can be explained by the presence of flamingos at the same time in the Çamaltı Saltern area. The saltpans of Çamaltı are the breeding site of flamingos and the site has become a regular and important breeding region in Türkiye since 2000 (Balkız et al. 2015). The greater flamingos can be observed in this area between March and August. Egg-laying generally occurs between mid-April and early May. Flamingo populations consist of 45% of ringed birds in Türkiye. These birds migrate to 16 different countries along the western and eastern Mediterranean Basin and West Africa. It has been reported that most of the Çamaltı flamingo population consist of migratory flamingos from the Mediterranean basins (France, Italy, Spain) (Balkız et al. 2015). It may be that the migratory dynamics of the flamingo population make it possible to transport cestode parasites between these basins.

We observed high cestode infections with high prevalence (up to 86.67±1.45%) between the late spring and summer season in the Çamaltı Saltwork populations of *A. parthenogenetica*. The mean intensity and abundance were calculated up to 1.92±0.06 parasite/per infected host and a mean abundance of 1.44±0.02 parasite/per investigated host respectively. Some former studies reported a lower prevalence rate than this study; Mura (1995) reported a cestode parasitism (*F. liguloides)* in an *Artemia* sp. population from south-western Sardinia with 3% prevalence and 1.09 mean intensity. Sánchez et al. (2013) reported cestode parasitism with 43% prevalence, 1.39 intensity and a 0.6 abundance score in June from the Odiel Saltpans - Southwest Spain, and with 63% prevalence, 1.53 intensity and 0.97 abundance in April from Salinas de Cerrillos-Spain. On the other hand, Georgiev et al. (2007) reported a cestode parasitism with high prevalence (89%) from Salinas Portuguesas, Cádiz Province, Spain. The parasitism intensity was a reported variable; less than 3 cysticercoids per individual in Algeria (Amarouayache et al.

2009), 13 in the populations of Spain (Georgiev et al. 2005) and around 9-11 in France (Gabrion et al. 1990). Sánchez et al. (2013) reported multiple cysticercoid infections of around 2-4, with a maximum of fourteen. At the start of September, salt harvesting begins in the Çamaltı saltworks, which means that the use of circulation pumps that provide water circulation in the ecosystem are discontinued. This means the period when all water inflows and outflows from the wetland cease. Therefore, the circulation of *Artemia* and the aquatic parasite end. Due to physicochemical and biological changes such as increasing salinity and decreasing nutrient content in the water, the possibility of encountering parasites in salt pans is negligible.

In this study, as in the results obtained by Koru in his 2018 study, a total of 7 and 4 cysticercoids were detected at most in adult and juvenile individuals, respectively (Koru 2022). According to Thiery et al. (1990), the accumulation of the cysticercoids would be associated with the age and the body size. It is indicated that *Artemia* larval instar III-IV can feed 25-30 µm diameter particles; however, *F. liguloides* eggs which are 40-50 µm in size may only be ingested by adult *Artemia* individuals (Mura 1995). The location of the cysticercoids was, for the most part, found in the thorax and abdomen in this study. However, they were typically located in the abdomen of adults in the saltworks of Sardinia (Mura 1995). Thiery et al. (1990) remarked that, the location of the cysticercoids is related to the volume of the hemocoel and the distribution is relevant to the allometric changes during the growth stage of the *Artemia*. In nature, individuals of *A. parthenogenetica* make vertical migrations; generally, are found 75% in the bottom of the water columns during the day and in the other 25% at night, (Sánchez et al. 2007) and generally shows strong negative phototaxy and positive geotaxis (Sánchez et al. 2007). Nevertheless, this infection changes the proportion of time that is spent at different depths (Sánchez et al. 2007, 2013). In addition, parasites increase the buoyancy and make *Artemia* swim on the surface of the water which facilitates predation by water birds (Thomas et al. 1997; Helluy & Holmes 2005; Curio 1988; Amarouayache et al. 2009). Sánchez et al. (2007) studied the effects of cestode parasitism on the behaviour of *A. parthenogenetica* and reported that 86% of the uninfected *Artemia* showed positive geotaxis whereas 53% of infected *Artemia* showed surface-swimming behaviour (negative geotaxis). Infected brine shrimps become photophilous and their surface-swimming movement can be observed. This action facilitates to be realized by the final avian host and make parasite transmission easier. Infected brine shrimps become photophilous and their surface-swimming action can be observed (Figure 6). Cestodes that are known to alter the action of their *Artemia* in ways that give the parasite a better home, or provide more nutrients, or cause the host to move to a different environment, and in doing so improve its own transmission, are going to be favoured by natural selection. Behaviours such as these mentioned would contribute to increasing the chance of the infected brine shrimp being consumed by a flamingo. In this study, the *F. liguloides* infestation which was observed in *A. parthenogenetica* as a vector demonstrates a similar example. Parasitic castration and colour change from transparent to red is known to be observed in infected brine shrimp to benefit the parasite in addition to behavioural manipulation making *Artemia* more susceptible to predators also hypothesize that the parasitic infection increases the brine shrimps' lifespan, time spent at the water surface, and that the castration is to prevent the shrimps from spending time with sexual reproduction (Amat et al. 1991; Rode et al 2013; Sánchez et al. 2006).

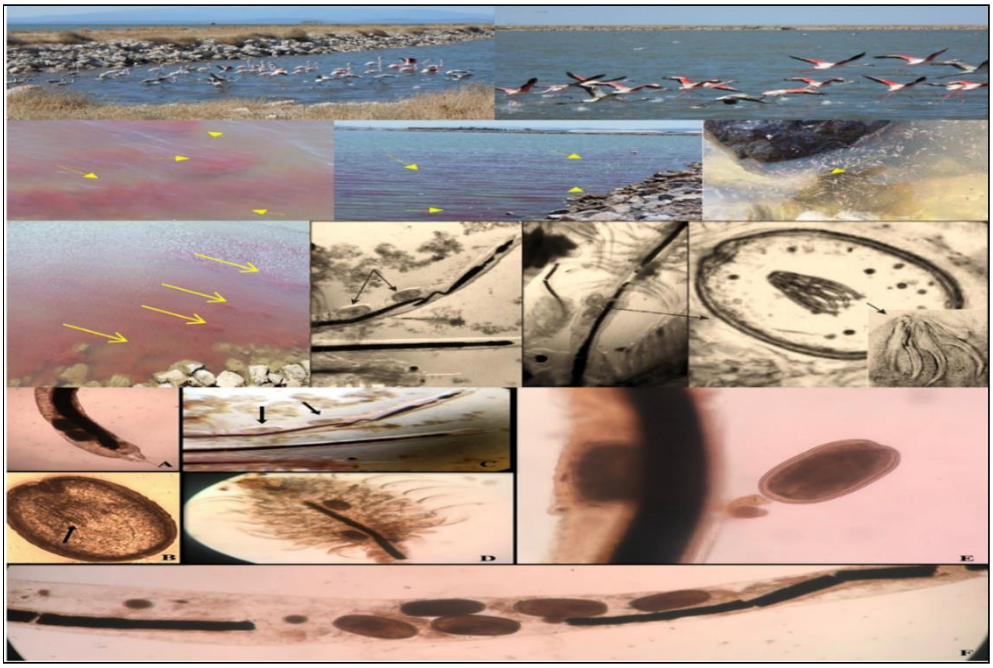

Figure 6- *Artemia parthenogenetica* individuals swimming towards the water surface infected with parasite carried by flamingo birds. (A-B-C-D-E-F). Cysticercoids of *Flamingolepis liguloides* (Gervais 1847) in the brine shrimp (*Artemia parthenogenetica*). A) Parasitic cyst in the *Artemia* caudal furca, B) Isolated parasitic cyst, C) Cysticercoid in the abdomen of the host, D) Cyts *of F. liguloides* in *Artemia* thorax, E) Cysticercoids of *F. liguloides* from *A. parthenogenetica* rectum, F) Parasitic cyts of *F. ligulepis* in *A. parthenogenetica* rectum (The photo was taken by Edis Koru in May-September)

5. Conclusions

Brine shrimp *Artemia* species are live food most commonly used in aquaculture production. It is the first nutrient in the larval stage of many aquaculture applications. Therefore, it is a zooplankton that should be used very carefully microbiologically. This species carries factors that can cause many aquaculture-related diseases. For this reason, it is necessary to know the factors that may cause pollution in the ecosystems in which *Artemia* grows. There is a strong link between parasitic parasitism in flamingos and *Artemia*. There is a noticeable connection based on nutrition between *Artemia*, at the bottom of the food pyramid, and the Flamingo, at the top. Through this relationship there is a risk of parasite contamination to fish with the relationship of *Artemia*, which is an important zooplankton in aquaculture nutrition. For this reason, biological events occurring in wetlands are and must continue to be researched and recorded. It is important to have data and records on all biological characteristics of a wetland that is the habitat of *Artemia*. The life cycle that takes place in wetlands every year depending on many natural factors shows us the situation of encountering different data. In addition, these data on the subject are among the first records of research findings on this geographical region of the Mediterranean (Çamaltı saltern). This is the first study on a *Flamingolepis* species infecting *A. parthenogenetica*, the parasite prevalence in Turkish wetland waters. In order to understand the effects of *F. liguloides* parasitism in the region, it is necessary to investigate the prey-predator relationships and their ecological effects. Saltworks are highly dynamic ecosystems in terms of biological and physicochemical properties. More multidisciplinary studies are needed to understand the parasitism cycle and the interaction between *Artemia* and flamingos in the Çamaltı wetland. Unfortunately, information regarding the population dynamics of *Artemia* in sampling areas in Türkiye is very limited. Comprehensive (such as salt production process, microalgae, zooplankton, benthic data, climate and atmospheric data) research over many years is needed to better understand the behavioral manipulation of this parasite and its effects on the interaction between *Artemia* and flamingos in the Çamaltı saltworks ecosystem.

Data availability: Data are available on request due to privacy or other restrictions

Conflict of Interest: No conflict of interest was declared by the author

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