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MEDITERRANEAN SEA FRY; A SOURCE OF ISOPOD INFESTATION PROBLEM IN EGYPT WITH REFERENCE TO THE EFFECT OF SALINITY AND TEMPERATURE ON THE SURVIVAL OF LIVONECA REDMANII (ISOPODA: CYMOTHOIDAE) JUVENILE STAGES.

By

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Abstract

Parasitic isopods have received considerable attention as they cause series impacts on their hosts either alone or with other environmental stresses such as water pollution. The present study investigated the infestation status with isopods among the fry collected from the natural sources .Also to demonstrate the effects of different salinity and temperature combination on the survival rates of the identified isopods under the laboratory conditions. In March 2017,a total of 16 thousand mugiliid fry were sampled from The Fry Collection center at Al Behera Governorate ,Egypt and examined for the presence of isopods .Samples were found infested with a rate of 10.6% , the result which practically documented that isopods were introduced into lake Qarun through dumping millions of infected fry from this source without biosecurity procedures. The isolated juvenile isopods were morphologically and molecularly identified *as Livoneca redmanii* species by using 16S rRNA gene and recorded in the GenBank with accession number: MK584629. The effects of variables of salinity/ temperature combination on the survival% of the identified juveniles showed that the best survival rate(100%) was recorded in salinity 15 -20% at temperature 25^{0}C while the lethal combination was in salinity 10, 20,50 and 60% at temperature 35^{0}C . Statistical analysis using A-two way ANOVA indicated a significant interaction between the effect of different degree of temperature and variance of salinity% on the survival of *L. redmanii* juveniles. Analysis of one way ANOVA indicated that the most effective temperature of overall mean temperature affected on survival of juvenile stage was 35 C^0 while the most effective salinity percentage on survival of Juvenile from overall mean of salinity % was 10% .Results of this study provided applicable procedures which can be recommended as a precaution measures and for eliminating the isopod juvenile stages among the mugiliid fry.

Key words: Mugliid Fry; Cymothoid isopod; *Livonica redmanii*; Salinity, Temperature.

Introduction

Cymothoid isopods are large fierce looking ectoparasitic crustaceans. They are protandrous hermaphrodites inhabiting brackish water, freshwater and marine environments (Tansel and Fatih, 2012). They are recorded worldwide and are mostly host and site specific (Saito et al 2014). Cymothoid species affect body surface, buccal cavity and branchial cavity of their host causing considerable tissue damages and even mortalities (Trilles and Bariche 2006).The life cycle of cymothoids is biphasic. Adult females are attached permanently to fish host and give birth to free-swimming mancae with six pairs of legs which developed into juvenile stages (Lindsay and Moran 1976) and (Sandifer and Kerby, 1983).

Infestation with isopod parasites was reported in marine Egyptian Coasts and Lakes (Noor El-Deen *et al,* 2013; Youssef *et al,* 2014; Maather and Abdel-Mawla, 2015; Abdel-Latif, 2016). In 2015, Lake Qarun (an inland closed salt water lake at Fayoum Governorate), which was exposed to a catastrophic invasion of several species of isopods causing fish mortalities and destroyied the fish stocks in the lake (Mahmoud *et al,* 2016; Elgendy *et al,* 2018; Helal and Youssef, 2018). The route by which isopods were introduced to Lake Qarun was questionable and needed practical field investigations to be proved. In Egypt, the sources of obtaining fry included hatcheries and natural

sources where the fry (mainly of mugiliid species) were captured from the seas and collected in Fry collection stations to be transported and officially delivered to supply the lakes annually. Studies for isopod control have been also conducted including chemical treatments (Shaheen *et al,* 2017) and biological trials (Mahmoud *et al,* 2017). Salinity and temperature are considered as the most important factors that physiologically affect the viability of marine organisms (Coineau, 1985; Aktas *et al,* 2004; Lemaire *et al,* 2002) and the degree of tolerance for both factors varied among different parasitic species particularly the crustaceans (Jansen, 1970; Jones 1972; Albuquerque, 2009; Thiyagarajan *et al,* 2003).

This study aimed to determine the infestation status of the mugiliid fry collected from the natural sources with isopod species. Also to demonstrate the effects of different salinity and temperature combination on the survival rates of the detected isopods under the laboratory conditions in order to record the most effective methods for controlling and preventing infestation spread through fry transportation.

Materials and methods

Collection of samples: In March 2017, a scientific documented field visits was done by the research team to the Fry Collection Station at Al Meadeyya Region, Al Beheira Governorate, Egypt (Fig. 1) where the mugiliid fry officially received to be transported to supply different lakes and fish farms. At the Station, a total of ten thousand randomly collected mugiliid fry units were examined on spot for the presence of isopod species. The fry were collected by fishermen by using nets of fine mesh size from the Boughaz Al Meadeyya (a channel of 20m wide, 100m long and 2m deep) in connection between Lake Edko and Mediterranean Sea Coast (Waheed *et al,* 2013) (Fig. 1 B). In this location, the temperature and salinity of water were measured in situ using portable optical TDS salinometer/refractometer. The isolated isopods and additional 6 thousand randomly collected fry samples within their original sub-surface water were transported in separate containers (F. 2B) supplied with oxygen to the laboratory as samples were kept in their original water in aerated glass aquaria for further investigations.

Morphological identification of the isolated isopod species: Isopods were measured to the nearest millimeters (mm), photographed using a digital camera (Cannon of 12 mega pixels) and morphologically identified according to (Bruce, 1990; Jones *et al,* 2008; Mahmoud *et al,* 2017)

Molecular identification: The DNA extraction from isolated isopods was extracted using DNeasy Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted DNAs were stored at -20°C till used. Extracted DNAs from Juvenile stages were using the primers, Fish-F1 (5`AGCCCTGTTCAATGGGATTA -3`) and Fish-R2 (5`TCCCTGGGGTAGTTTCATCTT -3`) to amplify a 417bp fragment of the 16S ribosomal RNA gene (Thangara *et al,* 2014). TPCR reaction volume was 25µl and each reaction mixture contained 12.5µl of 2X PCR Hot-StarTaq® master mix (Qiagen, Germany), 0.1µM of each primer, 1µl of DNA template and completed up to 25µl with nuclease free water. PCR assay was performed in T100™ Thermal Cycler (Bio-Rad, USA) under the following conditions: initial denaturation at 94°C for 2min followed by 35 cycles of 1min at 95°C, annealing at 54°C for 30sec, an extension at 72°C for 1min and then a final extension step of 10min at 72°C. Nuclease free water was used as a negative control.

DNA sequencing: PCR products of positive samples were purified using a QIA quick purification kit (Qiagen, Germany) for sequencing using Big Dye Terminator V3.1 kit in ABI 3500 Genetic Analyzer (Applied Biosystems, USA). The sequences were compared with those available in the GenBank using a BLAST server on the NCBI website. Nucleotide Sequence: partial sequences of the isopod species 16SrRNAgene were sub-

mitted to GenBank.

Experiment procedure: For demonstrating the impacts of the variable salinity/ temperature combination on the survival of the isolated isopod species under laboratory conditions, an experiment was designed following (Albuquerque *et al,* 2009) with some modifications. Groups of 15 isopods (in 3 replicates of 5 parasites each) were put in 250ml of the tested salinity in 500ml capacity glass beakers and exposed to variable salinity/temperature combination. Tested isopods were allowed one minute after exposure for adaptation with the surrounding, then the beakers were covered with transparent thin sheath (to reduce change of water salinity by evaporation) and supplied with an aerator tubes. The experiment included 5 temperature degree (15, 20, 25, 30 & 35^{o}C) and eight salinities (15, 20, 25, 30, 35, 40, 50 & 60%) to verify a total of 40 salinity/ temperature combination. Salinitiy less than the sea water were prepared using distilled water and higher one was by evaporating sea water at 50^{o}C. Salinity and temperature were monitored by using digital TDS salinometer/ refractometer. Beakers were examined after 6hr, 12hr, & 24hr of exposure then every 24hr till 72hr. Isopods were neither with visible movement nor response to the external touch by needle under binocular microscope were dead and removed (Johansen, 1999). Control groups were kept in salinity 33% and temperature 25^{o}C as these are the average that the isolated isopods were naturally exposed in field at time of collection. Statistical analysis: Numerical data for different grade of temperature, salinity and survival % were carried out using the two-way ANOVA test (Sokal and Rohlf, 1995). Further statistical analysis of the numerical differences in survival of the isopod species using variance of salinity percentages was conducted by using the SPSS 20.0 statistics software for determined significant differences at $P<0.05$.

Results

Examination of 16 thousand mugiliid fry randomly collected from the Al Meadeyya Fry Collection Station showed free swimming isopod stages with the rate of 10.6%.

The detected isopod species was identified taxonomically as *L. redmanii* juvenile stages (Fig. 2A). They were of body length ranged from 10-16mm (mean13mm) having pale translucent grayish color with brown chromatophores on the body dorsal aspect. The juvenile stage has large oval eyes, six pairs of legs and the pleopods provided with fine setae.

The isolated isopod species revealed amplicon with molecular weight 417bp. The sequence analysis explained that the samples were 96% identical with *Livoneca redmanii*. This identified species was recorded in the GenBank on the NCBI with accession number: MK584629.

Using of 40 salinity/temperature combination under laboratory conditions revealed that the survival rate of 100% for the juveniles of *L. redmanii* was recorded at temperature 15^{o}C, 20^{o}C & 25^{o}C where the tested juveniles able to resist salinity of 20% -40 % (till 72hr exposure). The lower lethal salinity of 50% of the tested juveniles (LS_{50}) was 20% at 20^{o}C while the higher LS_{50} was 25% at 30^{o}C (after 48hr). At temperature 30^{o}C, the mortality rate was 100% at salinity 10% and 60% (extreme salinities) after 24hr, while the juveniles resisted the salinity of 20% to 50% (survival rate ranged from 20-50 %). At temperature 35^{o}C, only 10% of the exposed juveniles can resist the salinity range of 20-40% while all juveniles (100%) died in the S 10%, 20%, 50% and 60%. Mortality was 0% in control group till the end of experiment (72hr), 6hr and 12hr of exposure for all combination and also after 48hr to 72hr exposure.

A two-way ANOVA was conducted to verify the effect of different grades of temperature and salinity level on the survival of the juvenile stage of *L. redman*ii under laboratory conditions. Result indicated a statistically significant interaction between the effect of different grading of temperature

and variance of salinity% on survival of the juvenile species, F (28, 80) =3.69, P= 0.00.

The effect of temperature, salinity and their interaction gave significant effect on the percentage of survival of juvenile stage of *L. redmanii* (P=0.00). It is important to concern by salinity*temperature interaction on survival of juvenile stages was zero at salinity* temperature interaction was 10% *30^{o}C, 60%*30^{o}C, 10%*35^{o}C, 20%*35^{o}C, 50%*35^{o}C and 60%*35^{o}C respectively (Tab.1, Ch. 1). Analysis of one way ANOVA using Duncan test explained significant difference between overall mean differences of temperature and salinity (P<0.05). The most effective temperature of overall mean temperature affected on survival of juvenile stage was 35^{o}C (10±2.63), but without significant difference at p<0.05 between effects at 15^{o}C & 25^{o}C (82.5± 5.81& 79.17±4.88 respectively).

The best salinity affected juvenile survival was 10% (20±10.9), but without significant difference between salinity 20% & 25% (54.67±17.17.1 & 58.67±14.8 respectively) and between 30% & 35% (70.67±17.9 & 69.33 ±17.4 respectively) at p<0.05 (Tab. 1, Ch. 1 & 2).

Table 1: Effects of variance of temperature and level of salinity % on survival of juvenile stage of *L. redmanii*

Salinity %	10	20	25	30	35	40	50	60	Overall mean of Temp.
Temp.	% of survival of juvenile stage of *Livonecaredmani*								
15	20±11.5^{a}	100±0.0^{c}	100±0.0^{c}	100±0.0^{c}	100±0.0^{c}	86.67±6.67^{bc}	86.67±13.3^{bc}	66.67±6.7^{b}	82.5±5.81^{D}
20	20±11.5^{a}	53.33±6.7^{b}	53.33±6.6^{b}	100±0.0^{d}	93.33±6.7^{d}	86.67±6.6^{cd}	80±11.5^{cd}	66.67±6.7^{bc}	69.17±5.64^{C}
25	60±11.5^{ab}	80±11.54^{bc}	80±11.5^{bc}	100±0.0^{c}	100±0.0^{c}	100±0.0^{c}	73.33±6.7^{bc}	40±11.5^{a}	79.17±4.88^{D}
30	0±0.0^{a}	40±11.5^{b}	46.67±13.3^{b}	26.67±6.6^{ab}	26.7±6.67^{ab}	33.3±6.67^{b}	20±11.5^{ab}	0±0.0^{a}	24.17±4.1^{B}
35	0±0.0^{a}	0±0.0^{a}	13.3±6.6^{ab}	26.67±6.7^{b}	26.67±6.7^{b}	13.3±6.6^{ab}	0±0.0^{a}	0±0.0^{a}	10±2.63^{A}
Overall salinity%	20±10.9^{a}	54.67±17.17.1^{cd}	58.67±14.8^{cd}	70.67±17.9^{e}	69.33±17.4^{e}	64±17.07^{de}	52±17.5^{c}	34.67±14.9^{b}	53±5.8

SE= Standard error, $^{a, b, c, d, e}$ Different superscripts within same raw of mean of survival of *L. redmanii* with significant difference at P< 0.05. $^{A, B, C, D}$ Different superscripts within same column (overall mean of temperature) indicate significant difference at P< 0.05.

Discussion

Examination of the mugiliid fry from the collection station revealed high infestation rate (10.6%) with free swimming juvenile isopods identified morphologically and molecularly as *Livoneca redmanii.* This species was recorded as the most abundant species invaded Lake Qarun (Mahmoud *et al,* 2017; Helal and Youssef, 2018). The present of high rate of isopods among the officially transported mugillid fry collected from the Mediterranean Sea confirmed that the invasive isopods were introduced into Lake Qarun through dumping fry from infested source without examination or biosecurity program. Among the examined fry, no adult stages of *L. redmanii* were recorded as the adult females and males of this species are mostly attached in pairs in the branchial cavity of their hosts (Sandifer and Kerby, 1983) .This result agreed with Eissa *et al*. (2012) and Kayiş and Ceylan (2011).

In the present study, *L. redmanii* juveniles showed moderate to high tolerance survival from 50 to 100%) of the tested species to the salinity between 20 to 60 % at temperature degree from 15 to 25^{o}C .The tolerance decreased (survival 0%) with increasing temperature (up to 35^{o}C) .The same agreed with Sjoeberg (1967) and Jansen (1970) for adults of the isopod *Jaera albifrons* and *Sphaeroma hookeri* respectively. Juveniles of *L. redmanii* resisted low temperature (15^{o}C) rather than high (35^{o}C) and tolerated better the salinity variation at low temperature. Also Kinne (1964) mentioned that temperature can change the salinity effect and vice versa. Death of the isopod juveniles might be due to the disturbance of their osmo-regulatory system (Lucu and Towels 2002).

The juveniles showed lowest survival% at high temperature in combination with extreme low and high salinity%. But, Albuquerque *et al.* (2009) found that for the isopod *Coxicerberus ramosae* adults, lowest survival rate was at low temperature/ low salinity combination. Variation might be due to species or stages difference (Charmantier and Charmantier-Daures, 1994) who found that euryhalinity increased progressively in

isopod juvenile stages to maximum in the latest stages and even up to adults. Other factors may also be the parasite size, water parameters or water pollution.

In the present study, the best combination for 100% survival of *L. redmanii* juveniles was at 15°C with salinity from 20 to 35%, at 25°C with salinity from 30 to 40% and at 20°C with salinity 30%. This showed a high level of euryhalinity for *L. redmanii* juvenile stages. No mortality after 6hr & 12hr in all combination after 48hr to 72hr of exposure showed that *L. redmanii* juveniles have high degree of adaptation so this was the cause of the isopod abundance in Qarun Lake environment.

Unfortunately, the natural field condition of salinity/ temperature during the mugiliid fry transportation season (from February to April) lies within the same range of the best combination for 100% survival of tisopod juvenile species that promoted the fish infestation within the water surfaces if received infested fry from natural resources specially that of salt or marine nature.

Conclusion

The present study practically proved that, infected fry from natural sources played the main role in transmitting isopod infestation. Also the results indicated that, the combination of salinity 10% / 35°C was of lethal effect on the isopod juvenile stages (100% mortality after 24-30 hours expos- ure), so exposure of the mugiliid fry to this lethal temperature/ salinity combination as a precaution measures and method of elimination of the isopod juvenile stages of *L. redmanii* among them was recommended to be applied.

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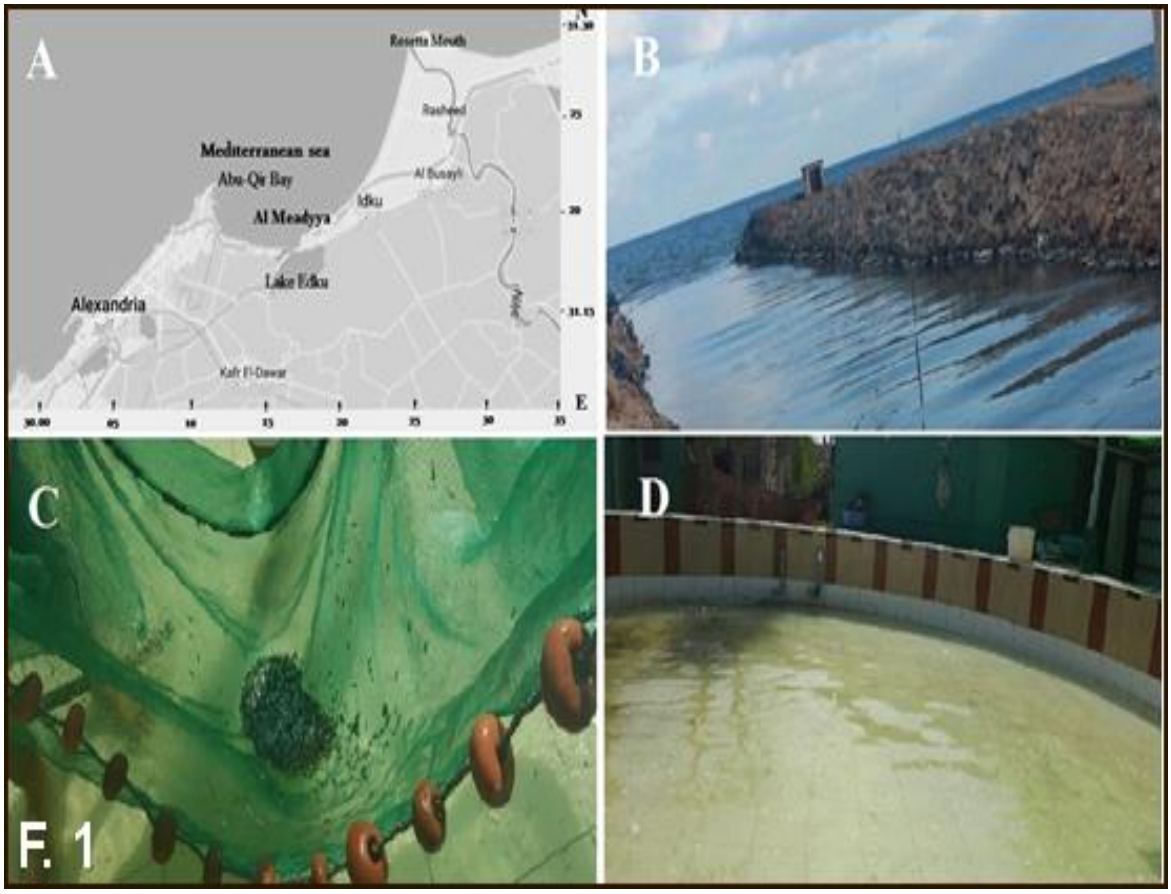
Explanation of figures

Fig 1: Locations, B Boughaz Al Meadeyya, C Collection of Mugliid fry in fine mish net D Basin inside the Fry Collection Station.
Fig 2: A dorsal and ventral aspect of *L. redmanii* juveniles; bar 1 cm B collected Mugliid fry samples
Chart 1: Effect of temperature, salinity and their interaction on percentage of survival of juvenile stage of *L. redmanii*
Chart 2: Mean survival of *L. redmani* juveniles at different temperature.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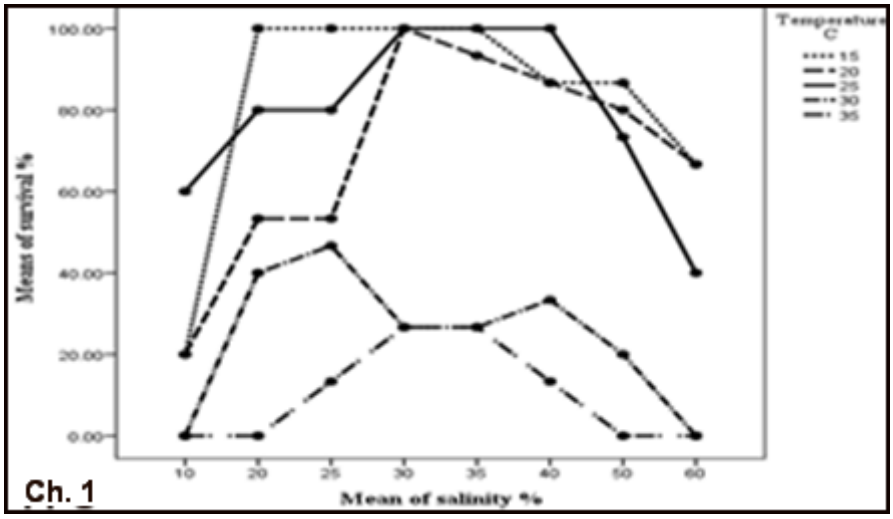
Temperature C
15
20
25
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35
Means of survival %
100.00
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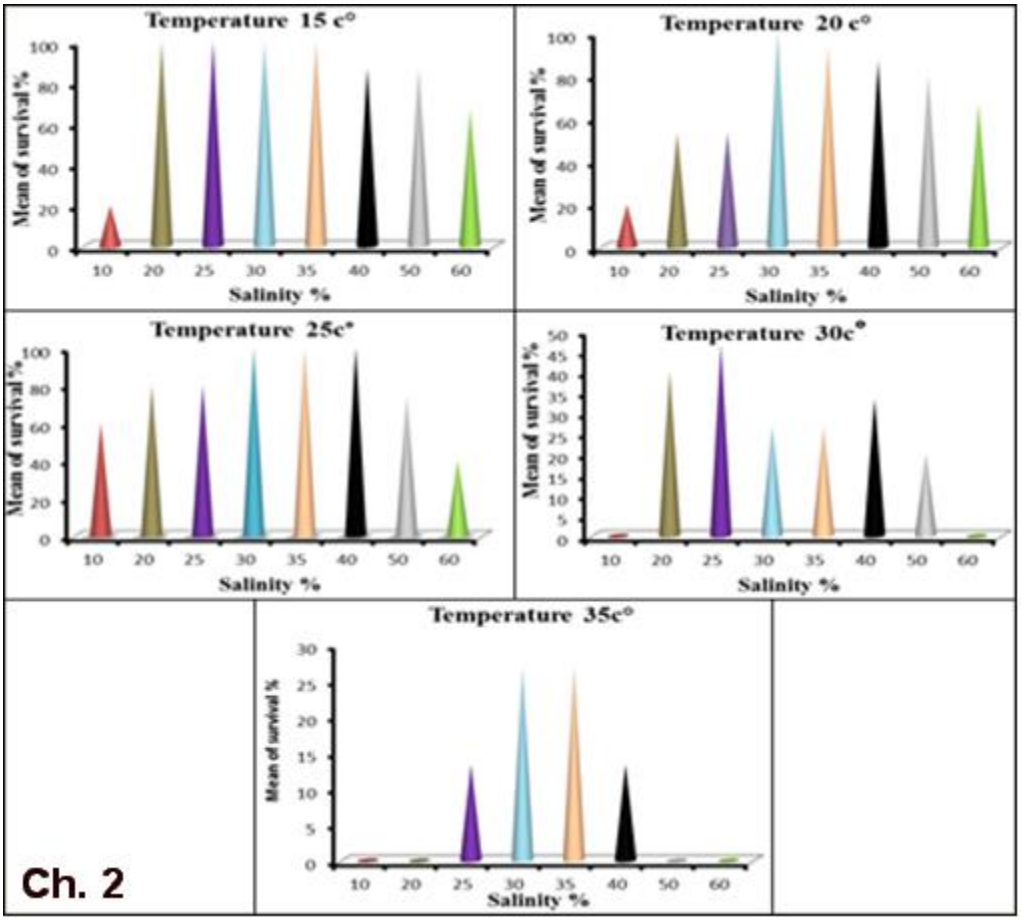
60
Mean of salinity %

Ch. 1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Temperature 35c°
Mean of survival %
Salinity %

Ch. 2