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ESTABLISHMENT OF JAVA MEDAKA (Oryzias javanicus Bleeker) AS TEST ORGANISM FOR ECOTOXICOLOGICAL STUDIES IN TROPICAL REGION

SHAHRIZAD BINTI YUSOF

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By

SHAHRIZAD BINTI YUSOF

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Chair : Ahmad Bin Ismail, PhD
Faculty : Science

The medaka fish is a group of small fish distributed in large areas in Asia. This study attempts to establish Java medaka (*Oryzias javanicus*) as a test organism for ecotoxicological studies in order to gain knowledge of the changes in biological processes of other organisms when exposed to pollutants. The ecology of Java medaka was investigated to understand their responses to environmental pollutants. The findings covered the distribution and localities of the fish, habitat preference or niche they occupied, relationship with other medaka species, and their spawning ground. Thirty eight localities in the coastal areas in Peninsular Malaysia were surveyed from 2008 to 2013. All localities surveyed in the west and south coast are inhabited by Java medaka in abundance but they do not occur in the east coast. In several localities, Java medaka co-exists with Indian medaka (*Oryzias dancena*), a phenomenon that has never been reported before. In other localities they inhabit different niche with Indian medaka, preferring areas with higher salinity while Indian medaka choose lower salinity areas. Java medaka can tolerate a wide salinity range (0.2 – 30.0 ppt), making them suitable to represent freshwater and marine environment. To support research activities using Java medaka, the laboratory culture was established. It is carried out in ambient temperature, thus, it can represent almost similar condition with the fish habitat. Their suitability for laboratory cultivation, ease of maintenance, regular spawning, short embryonic period and generation time make Java medaka a convenient test organism. The laboratory culture established in this study further ensures that the test fish is of high quality, free from parasites and diseases. The sensitivity of different life stages of Java medaka to environmental pollutants was tested. All life stages of the fish has been utilised and they have shown particular sensitivity. The embryos were sensitive to low concentrations (0.01 – 0.05 ppm) of heavy metals (Cd, Hg, Pb, Cu and Zn) in terms of developmental impairments. The most significant impairment was embryonic death which was metal dependent where the rank of metal severity is as follows: Cd (47.0 - 100%) > Hg (22.0 - 64.0%) > Pb (10.0 - 55.0%) > Zn (6.0 - 40.0%) >Cu (4.0

- 38.0%). In exposure to glyphosate-based herbicide, embryonic death was found to be directly proportional to the exposure concentrations. Other developmental endpoints in embryos exposed to the pollutants tested include embryotoxicity and teratogenicity. The developmental stages of Java medaka constitute simple and effective models for evaluating pollutant impact. The induction of micronucleus formation and nuclear abnormalities when the adult fish were exposed to very low levels of Cd and Hg (1.0 and 10.0 ppb) and the induction of only nuclear abnormalities in exposure to higher concentrations of Cu and Zn (10.0 and 100.0 ppb) showed the difference in their genotoxic responses towards different metals. Utilizing Java medaka in testing will closely reflect the impacts of toxic pollutants on the environment particularly for the tropical region. As the fish is new to many local researchers, the findings of this study provide the much needed information. Future researches can utilise the fish in various fields of scientific investigation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
Sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENUBUHAN JAVA MEDAKA (*Oryzias javanicus* Bleeker) SEBAGAI ORGANISMA UJIAN DALAM BIDANG EKOTOKSIKOLOGI

Oleh

SHAHRIZAD BINTI YUSOF

Jun 2014

Pengerusi : Ahmad Bin Ismail, PhD
Fakulti : Sains

Ikan medaka terdiri daripada sekumpulan ikan kecil yang tersebar di kawasan yang luas di Asia. Kajian ini bertujuan untuk menubuhkan 'Java medaka' (*Oryzias* javanicus) sebagai organisma ujian untuk bidang ekotoksikologi dalam usaha untuk mendapatkan maklumat tentang perubahan yang berlaku dalam proses biologi dalam organisma lain apabila terdedah kepada bahan pencemar. Ekologi 'Java medaka' telah dikaji untuk memahami gerak balas mereka terhadap bahan pencemar alam sekitar. Hasil kajian meliputi taburan dan lokaliti, pemilihan habitat dan nic, hubungan dengan spesies medaka lain serta tempat pembiakannya. Tiga puluh lapan lokaliti di kawasan pesisiran Semenanjung Malaysia telah dikaji dari tahun 2008 hingga 2013. Kesemua lokaliti yang dikaji di pantai barat dan selatan mempunyai taburan "Java medaka" yang tinggi tetapi ikan ini tidak terdapat di pantai timur. Dalam beberapa lokaliti, "Java medaka" wujud bersama "Indian medaka" (*Oryzias dancena*), suatu fenomena yang belum pernah dilaporkan sebelum ini. Dalam lokaliti lain mereka hidup dalam nic yang berbeza di mana "Java medaka" memilih kawasan yang mempunyai saliniti tinggi dan "Indian medaka" memilih kawasan bersaliniti rendah. "Java medaka" boleh hidup dalam julat saliniti yang besar (0.2 – 30.0 ppt), yang menyebabkan mereka sesuai untuk mewakili persekitaran air tawar dan marin. Untuk menyokong aktiviti-aktiviti penyelidikan menggunakan 'Java medaka', sistem pengkulturan ikan ini di dalam makmal telah ditubuhkan. Ia dijalankan dalam suhu ambien dan dengan itu dapat mewakili keadaan yang hampir sama seperti habitat ikan tersebut. Kesesuaian untuk dibiakkan di makmal, kemudahan penyenggaraan, kekerapan bertelur, tempoh perkembangan embrio dan generasi yang pendek menjadikan "Java medaka" organisma kajian yang sesuai. Kejayaan pengkulturan ikan ini di dalam makmal memastikan penghasilan ikan yang berkualiti tinggi, bebas dari parasit dan penyakit. Kepekaan dan respon oleh pelbagai peringkat hidup 'Java medaka' kepada beberapa bahan pencemar telah diuji. Semua peringkat hidup ikan ini telah digunakan dan mereka telah menunjukkan kepekaan tertentu. Embrio ikan ini bergerak balas kepada kepekatan logam

berat (Cd, Hg, Pb, Cu and Zn) yang rendah (0.01 - 0,05 ppm) dari segi gangguan perkembangan. Gangguan perkembangan yang paling signifikan ialah kematian embrio yang bergantung kepada jenis logam berat di mana turutan keseriusan adalah seperti berikut: Cd (47.0 - 100%) > Hg (22.0 - 64.0%) > Pb (10.0 - 55.0%) > Zn (6.0 - 40.0%) >Cu (4.0 - 38.0%). Dalam pendedahan kepada herbisid berasaskan glifosat, kematian embrio adalah berkadar terus dengan kepekatan herbisid. Gangguan perkembangan lain akibat pendedahan kepada bahan pencemar yang diuji termasuk keracunan dan kesan teratogenik. Peringkat awal perkembangan 'Java medaka' merupakan model ringkas dan berkesan untuk menilai kesan pencemaran. Aruhan pembentukan mikronukleus dan ketidaknormalan nukleus apabila ikan dewasa didedahkan kepada kepekatan Cd dan Hg yang sangat rendah (1.0 and 10.0 ppb) dan aruhan penghasilan nukleus tidak normal dalam pendedahan kepada kepekatan Cu dan Zn yang lebih tinggi (10.0 and 100.0 ppb) menunjukkan perbezaan dalam gerak balas genotoksik mereka terhadap logam berat yang berbeza. Penggunaan "Java medaka" dalam ujian akan menggambarkan dengan lebih dekat tentang impak bahan pencemar toksik terhadap persekitaran terutama di kawasan tropika. Oleh kerana ikan ini masih belum terkenal di kalangan penyelidik tempatan, dapatan dari kajian ini dapat memberikan maklumat yang sangat diperlukan. Ikan ini boleh digunakan dalam pelbagai bidang kajian saintifik.

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Ahmad Bin Ismail, PhD
Professor
Faculty of Science
Universiti Putra Malaysia
(Chairman)

Hishamuddin Bin Omar, PhD
Senior Lecturer
Faculty of Science
Universiti Putra Malaysia
(Member)

Siti Khadijah Binti Daud, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

_____

BUJANG KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ca^{2+}	Calcium ion
$CaCl.2H_20$	Calcium chloride dehydrate
Cd	Cadmium
cm	Centimetre
CO_2	Carbon dioxide
Cu	Copper
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
E	East
EPA	Eicosapentaenoic acid
F^-	Fluoride ion
Hg	Mercury
hr	Hour
KCl	Potassium chloride
km	Kilometre
L	Litre
LC50	Lethal concentration 50
$Lmin^{-1}$	Litre per minute
m	Meter
m^2	Meter square
Mg^{2+}	Magnesium ion
mgL^{-1}	Milligram per litre
MgSO4.7H2O	Magnesium sulphate heptahydrate

mm	Millimetre
N	North
N	Number of sample
N	Sample size
NaCl	Sodium chloride
^{o}C	Degree Celsius
POEA	Polyethoxylene amine
ppb	Parts per billion
ppm	Parts per million
ppt	Parts per thousand
SD	Standard Deviation
SE	Standard Error
Temp.	Temperature
UPM	Universiti Putra Malaysia
UV	Ultraviolet
Zn	Zinc
$ZnCl_2$	Zinc chloride
µg	Microgram

CHAPTER 1

INTRODUCTION

The release of many hazardous chemicals to the environment is currently identified as a global threat to human and other organisms□¶ealth as well as the environment itself. Yet, the world is continuously producing and releasing vast numbers and amounts of chemical compounds to the environment from time to time. The impacts of these chemicals on living organisms and the environment are not well studied much. These chemicals enter the environment, disperse, persist or bio-magnify through the food chain. They may enter the environment intentionally through measured release, for example the application of pesticides in agriculture activities or through unregulated release of industrial by products, household wastes and pharmaceuticals from hospitals. The increasing numbers of chemicals present in the environment due to human activities creates □D□□□J□O□R□E□D□O□□□ for researches on the impacts of these chemicals. In order to protect the environmental health, the possible biological consequences arise from the exposure and the accumulation of the pollutants needs to be strategically assessed. Many monitoring activities and reliable researches were established to evaluate the levels of these chemicals and their biological impacts.

Several organisms such as microorganisms, crustaceans, gastropods, worms and fish have been used either as indicators for these substances or as bio-monitoring agents or as test organisms. These organisms which act as biological indicators have been chosen by many scientists to measure environmental pollution instead of physical or chemical indicators. Physical and chemical indicators can neither show the cumulative effects of different pollutants in the ecosystem nor the duration of the existence of the pollutants which can be evaluated by biological indicators or bio-indicators (Karr, 1981). The application of biological methods can be integrated into other studies and does not cost much and the results can be obtained in a short time. Less equipment is required while a large area can be intensively surveyed and a large amount of information can be obtained for later assessment (Chapman, 1996). The effects of pollutants on the environment are often understood better when biotic factors are correlated with physical and chemical data. The organisms living in aquatic ecosystem reflect the history of the water body whereas chemical analysis is only valid for recent condition. Thus, biological assessment has become more popular and important globally with many methods have been developed.

As an indicator species, a bio-monitoring agent or a test organism should fulfil certain criteria. In order to obtain fast and comparable results, a responsive, readily available and representative organism is needed as test organism. The medaka fish is one of the most important model organisms among teleosts.

The medaka fish belongs to the genus *Oryzias* which is classified under family Adrianichthyidae. This family consists of a diverse group of small fish

that occupy the freshwater, brackish water and saltwater in large areas in Asia. The most established among the medaka fish is the Japanese medaka (*Oryzias latipes*) which has been widely used in experimental vertebrate biology for over a century. It is distributed in the freshwater of Japan, Korea and China (Naruse et al., 1993; Naruse, 1996). It is also a well established experimental organisms in fields such as embryology (Iwamatsu et al., 2005), physiology (Inoue and Takei, 2002) and molecular studies (Yamasaki et al., 2006; Imanishi et al., 2007). One of its relatives, the Java medaka (*O. javanicus*) is distributed in Peninsular Malaysia, Singapore, Indonesia, Thailand and Western Borneo (Iwamatsu et al., 1982; Roberts 1998; Magtoon and Termvidchakorn, 2009). Although Java medaka has only been used in scientific investigation recently, it is gaining reputation as an indicator species among the scientific community.

Java medaka also holds an important position in the estuarine food chain. Occupying the lower trophic levels, they are consumed by larger fish such as mudskipper and other important economic species. Their ecological role contributes to their importance in the aquatic ecosystem where they form a critical connection in the food web to larger fish, seabirds and marine mammals, thus creating a crucial ecosystem value. In addition, the coastal habitat in which Java medaka can be found includes coastline and mangrove areas that are of major economic importance and should be closely monitored and protected. Nevertheless, little is known on the ecology of Java medaka. In establishing an organism as test organism, information on the biology and ecology of the organism is very important in order to understand their responses to any tests or treatments. Sufficient knowledge on the biology and ecology of the organism would make the data from any tests easier to interpret. Thus, the first objective of this research is to investigate some ecological aspects of Java medaka.

To obtain consistent results from the experiments using the fish, a laboratory cultured Java medaka is required. Fish freshly sampled from the environment may not give uniform and comparable results due to their differences in age, size and the salinity of water where they are taken from. Furthermore, testing sensitivity may also differ between sampling locations due to prolonged conditioning to specific environment in the wild. This would make it very difficult for the local scientists in particular to make thorough assessment and to generalise the response shown by the fish in establishing a universal test organism. Therefore, the use of laboratory culture is very important as it could lessen the uncertainties brought about by these differences. Hence, the second objective of this study was to develop inexpensive, easy to maintain and sustainable culture and breeding technique for Java medaka in both saltwater and freshwater in the laboratory.

So far the utilisation of medaka as laboratory animal was carried out in places away from its natural habitat such as Japan, Korea, China and Taiwan. As the use of local or native species is one of the criteria for test organism it is very appropriate to establish our very own species as test organism for the tropical region. Moreover there is no perfect test organism been established yet to represent this type of ecosystem. As most of the

information and knowledge on both fish and its culturing system were developed and adapted to the different environments and seasons available in the said countries, establishing our very own culturing system is necessary. Developing the first medaka laboratory culture system in Malaysia, particularly in the Department of Biology, Universiti Putra Malaysia required systematic planning. Well-known medaka laboratories in Japan were visited to gain information on the laboratory culture for medaka fish. One of the key features that determine the success of the culture system lies in the manipulation of the physico-chemical factor which provides suitable condition for the fish to live healthily and breed at optimum rate. This is made possible through the information gathered while studying the ecology of Java medaka (the first objective of this study) which served as crucial knowledge for the culture establishment.

Java medaka is a small tropical fish closely related to the well-known Japanese medaka. This species has many characteristics similar to the well-established laboratory fish, Japanese medaka. They are hardy, have short life span and life cycle and grow very fast. They can be easily identified in their natural habitat and have a wide geographical range and availability. The high tolerance towards different salinity levels further distinguished the species from other bio-monitoring agents. This seawater and freshwater-adaptable species is suitable to be used to assess the health of aquatic systems in freshwater, estuarine, and marine environments. Nevertheless, there is no detailed information on laboratory culture methods and maintenance for Java medaka. While its foreign counterpart, the Japanese medaka has been extensively researched and utilised in laboratory for toxicology studies, Java medaka has the potential to be developed into a suitable test organism for the tropical region. Therefore, the information on the fish ecology in the various localities found in the wild would greatly aid in the development of the culture method and system for the fish in the laboratory.

There are growing numbers of studies that highlighted Java medaka responses to the various pollutants found in the natural environment (Imai et al., 2005; 2007; Koyama et al., 2006; 2008; Añasco et al., 2008; Cheikyula et al., 2008; Woo et al., 2006; 2009a; 2009b; 2014; Won et al., 2011; 2013; Khodadoust et al., 2013). A test organism for eco-toxicological studies should show excellent responsiveness to chemical contaminant exposure. Responses may either be acute or chronic and should be prominent and consistent throughout the different life stages of the fish population for particular doses or level of pollutants exposed. Therefore, the third objective of this study is to assess the fish capabilities to respond to hazardous chemicals in the environment in order to support the establishment of Java medaka as test organism.

In summary, in the establishment of Java medaka as a test organism for ecotoxicological studies, three objectives were specified:

i. To investigate several ecological aspects of Java medaka (*Oryzias javanicus* Bleeker).
ii. To develop a laboratory culture protocol for Java medaka.
iii. To assess the sensitivity of Java medaka to several environmental pollutants.

It is hoped that this study does not only provide fundamental information on the biology and ecology of the Java medaka in its native ranges, but it is also the first to highlight the establishment of laboratory culture system for Java medaka in its native country. This study utilised the knowledge on the biology and ecology of the fish to establish the culture system. Products from the laboratory culture were then utilised in exposure tests using several pollutants common to the local environment. Different life stages of the fish, from embryonic to adult were employed in the tests. It is a great hope that the findings will pave the way towards establishing our own medaka as sentinel test fish for tropical countries around the world.

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