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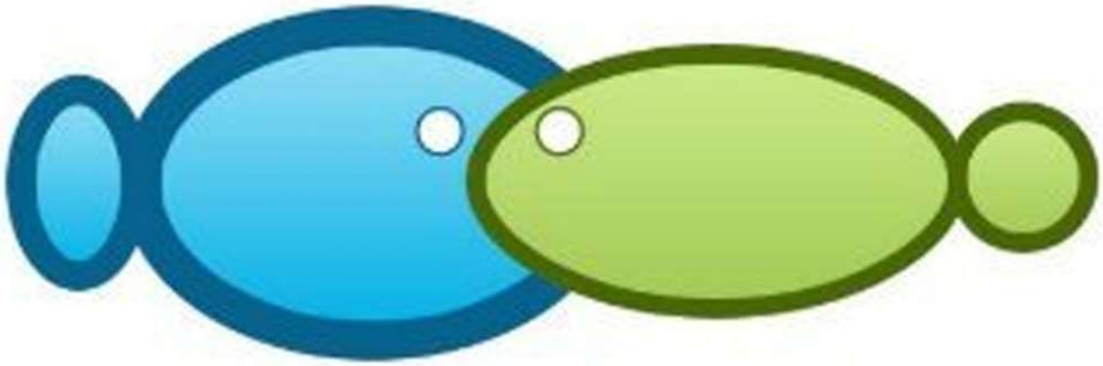

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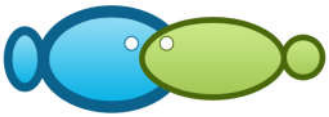

Contents

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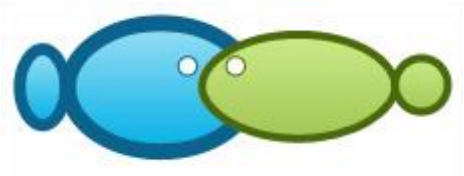

Enrichment of nutrition of *Brachionus* spp. in the tropical areas

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Abstract. Larvae feeds incorporating *Brachionus* spp. play a great role in fish cultivation. However, the development and nutritional content of *Brachionus* spp. depends on the nutrient enrichment provided. This study aims to determine the best nutrient enrichment for *Brachionus* spp. cultivated in the tropical areas. This research applied an experimental method with a completely randomized design (CRD) with 5 treatments and 4 replications. The data was analyzed with ANOVA and Duncan's multiple range test. The five nutrient enrichments used are as follows: *Chaetoceros* spp., *Tetraselmis* spp., yeast, *Chaetoceros* spp. + yeast, *Tetraselmis* spp. + yeast. From the treatment groups, the nutrient enrichment using *Chaetoceros* spp. resulted in the highest population, producing 97 ind mL^{-1}. The treatment group with the nutrient enrichment using *Chaetoceros* spp. + yeast presents the highest protein content, of 14.52%. The best nutrition for *Brachionus* spp. cultivated in the tropics are feeds made of *Chaetoceros* spp., according to the results.
Key Words: *Brachionus* spp., *Chaetoceros* spp. *Tetraselmis* spp., yeast.

Introduction. Natural feed is a basic requirement in aquaculture of both fish and shrimp. The availability of seeds for cultivation is influenced by the availability of feed at the seed stage. However, in some cases, the natural feed is not sufficient for the initial stages of the fish larvae. This requires farmers to continuously find innovations, so that the availability of natural feed for seeds or substitutes becomes more adequate both in terms of quantity and in terms of quality.

Brachionus spp. is a natural feed from the rotifer group that is often used in aquaculture. *Brachionus* sp. has several advantages as a natural fish feed, including its small size, slow swimming, easy breeding, and high nutritional value, all helping the feeding of fish (Sartika et al 2013). However, the production of nutritious rotifers can depend on the production of microalgae or phytoplankton they feed on (Lubzens et al 2001).

Brachionus spp. has 26-30% crude protein and 9-28% crude fat (Lubzens & Zmora 2003). In an experiment conducted by Xu & Pan (2014) on white shrimp (*Litopenaeus vannamei*), it was found that the optimal protein diet rate for shrimp growth performance and cost efficiency was 32.9%. Zang et al (2013) showed that an optimum dietary fat content for *L. vannamei* is around 10-12% for optimum growth performances. This shows that *Brachionus* spp. requires feed enrichment to increase the nutritional content, especially protein.

Sometimes the availability of *Brachionus* spp. is in lower numbers, and it is necessary to find alternatives to natural feed. There are few alternative feeds that can replace *Brachionus* sp. as the initial feed for larvae (Hagiwara et al 2001; Yoshimatsu & Hossain 2014), since it is a great source of nutrients and improves growth (Andriyono et al 2015). In some cases, *Brachionus* spp. requires enrichment to increase its nutritional content for larvae feed. There are various kinds of emulsion used for *Brachionus* spp. enrichment, which generally contain fatty acids. Fat is a high-energy component in fish feeds, while protein is needed for growth. However, the energy for metabolic processes comes from fat and carbohydrates. Protein is an organic compound with a high molecular

weight. It is composed of C, H, O, and N, as well as other elements, such as P and S, forming amino acid units (Sorgeloos et al 2001).

The enrichment increases nutrient levels from natural feeds to approach or even reach the nutritional needs of aquaculture species. Enrichment with microalgae can increase the nutritional content of rotifers. Some types of microalgae are often used as food for *Brachionus* spp., such as *Tetraselmis* sp., *Nannochloropsis* sp., *Chaetoceros* sp., *Rhodomonas* sp., and *Isochrysis* sp. (Dhert et al 2001; Wikfors & Ohno 2001).

This rotifer can present better growth if administered the suitable feed for its development. Therefore, this research was conducted to determine a nutrition enrichment that can provide a higher protein content for the population growth of *Brachionus* spp. cultivated in the tropics.

Material and Method

Research design and samples. This research was conducted at the Brackish Water Aquaculture Center, Situbondo (BPAP Situbondo), East Java, Indonesia, from October to November 2017. The study was conducted using an experimental method to determine the effect of administrating *Tetraselmis* spp., *Chaetoceros* spp., yeast, yeast in combination with *Tetraselmis* spp., as well as yeast in combination with *Chaetoceros* spp. to a population of *Brachionus* spp. The research applied a completely randomized design (CRD), where all the experimental units were in the same conditions with different treatments (Montgomery 2001). Furthermore, the population density of *Brachionus* spp. was observed in each treatment.

The research materials used were yeast, seawater, chlorine, formalin, sodium thiosulfate, lights, Guillard fertilizer, Walne fertilizer, *Chaetoceros* spp., *Tetraselmis* spp. and *Brachionus* spp. This study used five treatments and four replications. *Brachionus* spp. seed starters were cultivated in containers with a volume of 300 mL with a density of 10 ind mL^{-1}.

Tetraselmis spp. was provided in a density of $1x10^6$ cells mL^{-1} and *Chaetoceros* spp. was provided in a density of $3x10^6$ cell mL^{-1} (Sutomo 2007). The dosage of yeast in the *Brachionus* spp. culture is equivalent to the administration of *Tetraselmis* spp. and *Chaetoceros* spp. The treatments applied in this study are:
Treatment A: *Chaetoceros* spp. with a density of $3x10^6$ cell mL^{-1};
Treatment B: *Tetraselmis* spp. with a density of $1x10^6$ cell mL^{-1};
Treatment C: yeast (0.002 g);
Treatment D: *Chaetoceros* spp. with a density of $1.5x10^6$ cell mL^{-1} and yeast (0.001 g);
Treatment E: *Tetraselmis* spp. with a density of $5x10^5$ cell mL^{-1} and yeast (0.001 g).

Experimental diagram. The placement pattern of the treatment containers was carried out randomly, as presented in Figure 1.

A1	B1	E4	C2	C4
D1	E1	C1	B4	A2
B2	D4	A3	E3	D2
C3	A4	B3	D3	E2

Figure 1. The placement of containers with different treatments.

Parameter tests. The main parameters monitored in this study are population growth and the protein content of *Brachionus* spp. The observations were conducted every day for 12 days. Population growth was calculated using a Sedgewick Rafter counting chamber with a microscope (100X), and a hand tally counter. Samples of *Brachionus* spp. were collected to analyze the protein levels using the Kjeldahl method at the Situbondo BPBAP Nutrition Laboratory.

The supporting parameters in this study are temperature, salinity, ammonia levels, and dissolved oxygen (DO). Temperature was measured using a thermometer, pH was measured using a pH meter, salinity was determined with a refractometer, the DO was measured using a DO meter, and ammonia levels were determined using an ammonia test kit. Temperature measurements were conducted twice a day, while salinity and pH measurements were carried out once a day. The measurements of DO and ammonia were carried out at the beginning and at the end of the experiment. Supporting parameters were used to complete the main parameter data.

Statistical analysis. Data from the results of this study were analyzed using ANOVA (Hestianah et al 2014). The data was analyzed through the SPSS version 16.0 software. When the results showed differences, further testing was conducted using Duncan's multiple range test to determine differences (Alamsjah 2010).

Results and Discussion

The growth of Brachionus spp. A population increase of *Brachionus* spp*.* was observed every day for 12 days. The results of the observations are presented in the form of densities of *Brachionus* spp*.* (Figure 2). The results of the ANOVA analysis are presented in Table 1. They show that different feeds significantly affected the population density of *Brachionus* spp. Each treatment had an influence on the growth of *Brachionus* spp.

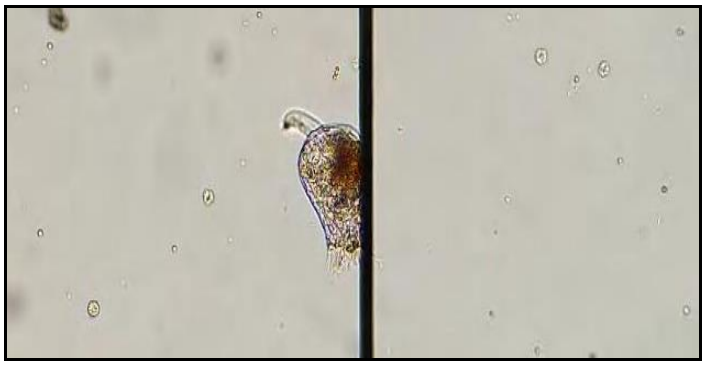

Figure 2. *Brachionus* spp (100X)*.*

Table 1

Brachionus spp*.* population growth results (ind mL^{-1})

Observation day	*Brachionus spp. (ind mL^{-1}) population numbers*				
	A	*B*	*C*	*D*	*E*
0	10.00	10.00	10.00	10.00	10.00
1	7.25^{b} ±1.89	12.50^{a}±2.38	7.50^{b}±0.57	12.25^{a}±1.70	9.75^{ab}±2.06
2	10.00^{a}±1.63	12.25^{a}±2.06	9.25^{a}±1.70	12.00^{a}±2.16	11.75^{a}±2.36
3	18.50^{a}±2.38	21.50^{a}±2.51	12.50^{b}±2.88	20.25^{a}±2.16	22.00^{a}±3.30
4	22.00^{ab}±3.46	23.75^{ab}±3.30	20.00^{c}±0.81	26.50^{a}±1.91	22.75ab±3.82
5	37.50^{a}±4.12	30.25^{b}±1.25	16.25^{d}±2.08	32.00^{b}±5.09	22.25^{c}±3.86
6	97.00^{a}±8.04	43.75^{c}±3.40	14.25^{d}±1.50	75.00^{b}±4.54	43.50^{c}±4.65
7	67.25^{a}±3.30	32.25^{c}±3.59	11.50^{d}±2.64	52.50^{b}±6.24	28.50^{c}±3.41
8	32.50^{a}±4.93	20.50^{c}±1.73	10.00^{d}±0.81	26.75^{b}±3.20	19.00^{c}±1.41
9	26.50^{a}±5.91	15.75^{b}±1.50	7.75^{c}±2.21	19.00^{b}±2.58	15.00^{b}±2.58
10	13.25^{a}±2.21	10.00^{b}±0.81	6.50^{c}±1.29	11.00^{b}±0.95	10.25^{b}±1.41
11	10.00^{a}±1.41	8.75^{b}±0.95	5.00^{c}±0.81	10.00^{b}±1.82	9.00^{b}±1.41
12	12.25^{a}±1.70	8.50^{b}±2.38	2.75^{c}±0.95	9.00^{c}±1.73	6.50^{b}±1.41

Note: Different superscript letters in the same column show significant differences ($P<0.05$). Treatment A - *Chaetoceros* spp. with a density of $3x10^{6}$ cell mL^{-1}; treatment B - *Tetraselmis* spp. with a density of $1x10^{6}$ cell mL^{-1}; treatment C - yeast (0.002 g); treatment D - *Chaetoceros* spp. with a density of $1.5x10^{6}$ cell mL^{-1} and yeast (0.001 g); treatment E - *Tetraselmis* spp. with a density of $5x10^{5}$ cell mL^{-1} and yeast (0.001 g).

Treatment A produces the highest population on day 6, with 97.00±8.04 ind mL^{-1}. Treatment B also produced the highest population on the 6th day, with 43.75±3.40 ind mL^{-1}. Treatment C presented the highest population on day 4, with 20.00±0.81 ind mL^{-1}, while treatments D and E presented highest densities in day 6, with 75.00±4.54 ind mL^{-1} and 43.50±4.65 ind mL^{-1}, respectively. The results from treatment A and D were significantly different from those of treatment C, but not significantly different from those of treatments B and E. The population growth from treatments B and E were not significantly different from that of treatment C.

The population growth patterns of *Brachionus* spp*.* (Table 1) presents an adaptation phase, a logarithmic growth phase and a death phase. The time needed to achieve the optimal rotifer population growth also varies for each type of feed used. In contrast to treatments A, B, D and E, which undergo an adaptation phase from the first day to the 4th day, C treatment undergoes an adaptation phase from the first day to the 3rd day. On the 4th day, treatment C experienced a growth peak phase, which decreased until the 12th day. Treatments A, B, D and E begin to enter the logarithmic growth phase on day 5 and reach the peak of the population on day 6, experiencing a phase of decline from day 7 to 12. The number of *Brachionus* spp*.* in each treatment increased until it reached its peak on the sixth day, excepting treatment C, which experienced a peak on the fourth day. This is presumably due to the inedible yeast C that caused a decline of environmental conditions and interfered with the maintenance process.

As in other studies, the increase in plankton population is visible in each day. *Brachionus* spp*.* fed with *Tetraselmis* spp. and *Chaetoceros* spp. developed proportional quality and an ever-increasing amount of nutrients (Ortega-Salas & Reyes-Bustamante 2013).

The highest average increase of *Brachionus* spp*.* population can be found in treatment A, with *Chaetoceros* spp., probably due to the fact that it is easily digestible. Biologically, *Chaetoceros* spp. is included in the class of diatoms that live in marine waters. Its exterior is covered by a shell from silicates with irregular geometric shapes (Hourmant et al 2009). Diatomic plankton is easily digested by zooplankton or fish (Sutomo 2007). *Chaetoceros* sp. is a diatomic plankton group containing β-carotene, thus being suitable for fish cultivation (Helm & Bourne 2004).

Treatment D shows an increase in the *Brachionus* spp*.* population that reached the peak of population on the sixth day, with a density of 75 ind mL^{-1}. The combination of microalgae and yeast can provide a sufficient population increase, good protein content, being also easily digested by the zooplankton group. These make *Chaetoceros* spp. a suitable feed for *Brachionus* spp*.* in combination with yeast. Combinations of microalgae and yeast have a positive effect on nutritional value, and can increase the growth and survival rate of rotifers (Sahandi & Jafaryan 2011). *Chaetoceros* spp. has good visibility, large size, and low ciliary contamination (Nhu 2004), being suitable as feed for rotifers.

The results of treatment B and treatment E indicate that significant differences are absent between the two treatments. The growth of *Brachionus* spp*.* with *Tetraselmis* spp. experienced a significant increase due to the density of food produced. This result is in line with the results of Rahman et al (2018), which measure rotifer growth rate (*Brachionus* spp.) fed different microalgae, such as *Nannochloris* sp., *Tetraselmis* sp., *Isochrysis* sp., *Chlorella* sp., and *Nannochloropsis* sp. in a density of 0.1×10^6 cells mL^{-1}). *Tetraselmis* sp. produced the highest growth rate value compared to other microalgae ($p < 0.05$), followed by *Tetraselmis* sp., *Isochrysis* sp., *Chlorella* sp., *Nannochloris* sp., and *Nannochloropsis* sp., with 1.40, 0.5, 0.24, and 0.1 cell mL^{-1}, respectively. However, their performance is still less productive compared to that of *Chaetoceros* spp. in regards to the cultivation of *Brachionus plicatilis* in the tropical areas.

Protein content of Brachionus spp. After finding the best nutrient enrichment that can increase the population of *Brachionus* spp. in a short period of time, the following step was to test the protein content of *Brachionus* spp. The crude protein content from *Brachionus* spp*.* is presented in Table 2.

The crude protein content of *Brachionus* spp*.* in treatment C is the lowest (5.13%) among the treatments. Thus, the sole administration of yeast did not work optimally in producing rotifer growth. The use of yeast in various densities results in similar

population growth. A combination of yeast and *Chlorella* sp. resulted in a maximum population increase of 25 ind mL^{-1}, which is considered a slow production (Khatun et al 2014). *Brachionus* spp. fed with yeast are unstable, have low nutritional value, and do not support high productions. Chilmawati & Suminto (2010) also state that yeast without the addition of supplements lacks the nutrients for the population growth of *B. plicatilis.*

Table 2

Crude protein levels of *Brachionus* spp. in different treatments

No	*Treatment*	*Crude protein content (%)*
1	A (*Chaetoceros* spp.)	11.15
2	B (*Tetraselmis* spp.)	5.81
3	C (*Saccharomyces* spp.)	5.13
4	D (*Chaetoceros* spp. + *Saccharomyces* spp.)	14.52
5	E (*Tetraselmis* spp. + *Saccharomyces* spp.)	10.29

Water quality. The water quality parameters are presented in Table 3. Water quality parameters during the study were still in a threshold suitable for the life of microalgae and *Brachionus* spp., except in treatment C, where ammonia was at the upper limit for *Brachionus* spp. life, which is 1 mg L^{-1}. Ammonia values during the study ranged from 0.003-1 mg L^{-1}. According to Fulks & Main (1991), the ammonia value in *Brachionus* spp culture should not exceed 1 mg L^{-1}. The high value of ammonia in treatment C is thought to be caused by dead and decayed yeast.

Table 3

Water quality during the eperiment

Water quality parameters	*Value range*				
	A	*B*	*C*	*D*	*E*
Temperature (^{O}C)	24.5-29.5	25.0-29.5	25-30	24.5-30	25.0-29.5
pH	7.4-8.2	7.5-8.2	7.5-8.2	7.5-8.2	7.5-8.2
Salinity (ppt)	29-32	28-32	28.0-30.0	29-32	28-32
Ammonia (mg L^{-1})	0.003-0.5	0.003-0.5	0.003-1.0	0.003-0.5	0.003-0.5
DO (ppm)	3.9-6.5	3.9-6.3	3.8-6.4	3.8-6.4	3.9-6.6

Note: DO - dissolved oxygen. Treatment A - *Chaetoceros* spp. with a density of 3x106 cell mL^{-1}; treatment B - *Tetraselmis* spp. with a density of 1x106 cell mL^{-1}; treatment C - yeast (0.002 g); treatment D - *Chaetoceros* spp. with a density of 1.5x106 cell mL^{-1} and yeast (0.001 g); treatment E - *Tetraselmis* spp. with a density of 5x105 cell mL^{-1} and yeast (0.001 g).

Water quality during the study in all treatments was between the limits suitable for the life of *Brachionus* spp. and microalgae. Water temperature values during the study ranged between 24.5-30^{O}C. According to Fukusho & Okauchi (1982), the optimum temperature for *Brachionus* spp. is between 25-35^{O}C. Values of pH during the study ranged from 7.5 to 8.5. According to Fulks & Main (1991), the pH values that can be tolerated by *Brachionus* spp. are between 5 and 9. Water salinity during the study ranged from 28-32 ppt. Dissolved oxygen content rangeed from 3.9 to 6.6 ppm. According to Effendi (2003), the DO level should be above 5 ppm.

Conclusions. The provision of different feeds and their combinations can increase population growth and crude protein content of *Brachionus* spp. For *Brachionus* spp. cultivated in the tropics, *Chaetoceros* spp. is the best feed out out the tested ingredients, because it contributes to the largest population growth, from a density of 10 ind mL^{-1} to 97 ind mL^{-1} or 970% on the sixth day. The highest crude protein content of *Brachionus* spp. (14.52%) was obtained in treatment D, with a combination of *Chaetoceros* spp. and yeast.

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SURAT KETERANGAN

Nomor: 1434/UN3.FPK/KP/2023

Yang bertanda tangan di bawah ini:

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Dengan ini menerangkan bahwa:

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Telah melakukan penelitian pada bulan Oktober tahun 2017 dengan judul sebagai berikut:
Enrichment of nutrition of Brachionus* spp. *in the tropical areas

Adapun penelitian ini sudah mengacu pada prosedur pertimbangan etik dari:

1. *American Fisheries Society* (AFS, 2014) yang berjudul *Guideline for the Use of Fishes in Research* yang menyebutkan bahwa: penelitian dalam kondisi laboratorium baru mengatur tentang hewan percobaan berupa ikan hidup, untuk hewan percobaan berupa zooplankton tidak termasuk (hal 43 ; terlampir), dan
2. *Canadian Council on Animal Care* (CCAC, 2005) yang berjudul *Guideline on the Care and Use of Fish in Research, Teaching and Testing* yang menyebutkan bahwa: pedoman tersebut hanya digunakan untuk hewan uji berupa ikan (Kelas: Chondrichthyes, Agnatha, dan Osteichthyes) dan Avertebrata (Kelas: Cephalopoda) (hal 13,14 ; terlampir).

Sedangkan dalam penelitian tersebut menggunakan zooplankton (*Brachionus* spp.) sebagai hewan percobaan. Sehingga penelitian tersebut tidak perlu dilakukan ***Uji Ethical Clearence.***

Demikian Surat Keterangan ini kami buat untuk dapat dipergunakan sebagai persyaratan pengusulan Jabatan Fungsional Guru Besar atas nama Dr. Endang Dewi Masithah, Ir., MP.

Surabaya, 27 April 2023

Mengetahui,	
Dekan FPK Unair	Wakil Dekan III FPK Unair
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Guidelines for the Use of Fishes in Research

Use of Fishes in Research Committee members:
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7. Laboratory Activities

7.1 General Principles

Working with live fishes under laboratory conditions requires attention to many details concerning the requirements for, and limits of tolerance of, the particular species under study. Acceptable physical facilities and an adequate supply of water with good quality must be provided, even if the fishes are to be held for only short periods of time. Although fish may tolerate marginal facilities and conditions for a few hours or even several days, holding them under less than optimal conditions will affect the results of the research. Standards for humane treatment of animals must also be maintained, regardless of the length of time that the fishes are held.

The reader should note that some content of section 7 is not restricted to laboratory activities, but may be applicable to field situations, as well.

7.2 Confinement, Isolation, and Quarantine

Prior to bringing fishes into a laboratory, facilities and plans should be in place to ensure that the fish cannot escape, especially species not native to the watershed, and that the introduced fishes can be isolated physically from fishes already present. Each holding unit should have its own set of nets and other equipment. Facilities and equipment used for previous studies should be disinfected prior to use in new studies, typically with a chlorinated disinfectant or another disinfectant such as Virkon® Aquatic (www.wchemical.com/). If the introduced fishes may carry disease agents, especially pathogens or parasites that are not endemic to the area, quarantine-level facilities should be used. The level of quarantine required will vary with the seriousness of the known or suspected disease agent (see section 2.5 Fish Health Management: Control of Pathogens and Parasites).

Individual fish with suspected ill health should be quarantined from the others so as to negate the potential for spread of potential disease agents. Such fish should be evaluated by an individual with expertise in fish diseases (fish pathologist or veterinarian), and the proper therapeutant should be applied as directed. Providing guidance for the treatment of specific diseases is beyond the scope of this document. The investigator is strongly urged to establish a working relationship with individuals with expertise in fish health with whom they may consult.

Experimentation with nonindigenous fishes, transgenic fishes, or other genetically modified fishes is a special situation that requires additional precautions to preclude their escape. Permitting with site visits by state wildlife agencies may be required for holding nonindigenous species (see section 3.4 Permits and Certificates). The specific barriers may be similar to those used to prevent the escape of disease agents but must be developed to fit the physical characteristics of the laboratory or experimental facility. The USDA has developed

Canadian Council on Animal Care

guidelines on:

the care and use of fish in research, teaching and testing

This document, the CCAC *guidelines on: the care and use of fish in research, teaching and testing*, has been developed by the *ad hoc* subcommittee on fish of the Canadian Council on Animal Care (CCAC) Guidelines Committee.

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the care and use of fish in research, teaching and testing

A. PREFACE

The Canadian Council on Animal Care (CCAC) is the national peer review agency responsible for setting and maintaining standards for the care and use of animals used in research, teaching and testing throughout Canada. In addition to the *Guide to the Care and Use of Experimental Animals*, vol. 1, 2nd ed., 1993 and vol. 2, 1984, which provide the general principles for the care and use of animals, the CCAC also publishes detailed guidelines on issues of current and emerging concerns. The CCAC *guidelines on: the care and use of fish in research, teaching and testing* is the seventh of this series. This document supersedes Chapter I - Fish, *Guide to the Care and Use of Experimental Animals*, vol. 2 (CCAC, 1984).

These guidelines aim to provide information for investigators, animal care committees, facility managers and animal care staff that will assist in improving both the care given to fishes and the manner in which experimental procedures are carried out.

The present document has drawn substantially from the work of organizations listed in Appendix A. Their contributions to the development of these guidelines are gratefully acknowledged.

The guidelines have been developed by the CCAC subcommittee on fish and were reviewed by a total of 69 experts. A preliminary first draft was agreed on by the subcommittee and circulated to experts in June 2002 (including representatives of the organizations listed in Appendix A), and a second draft was circulated for widespread comment in June 2003. A final review was carried out in August 2004 involving all individuals who had previously provided significant input to the development process. The development of these guidelines also involved consultation with the Canadian Association for Laboratory Animal Science (CALAS) and the Canadian Society of Zoologists (CSZ) through workshops held at annual meetings in Québec City (June 2003), Acadia University (May 2004), and Hamilton (June 2004). Consultations were also held at the Aquaculture Association of Canada and AquaNet annual meetings in Québec City (October 2004), and at the CCAC Workshop on the Fish Guidelines in Vancouver (April 2005).

The guidelines have been organized in a format that should facilitate easy access to relevant sections. Early sections provide an ethical overview relevant to the use of fishes in research, teaching and testing. This is followed

by a brief overview of regulations and responsibilities relevant to the care and use of fishes in science in Canada. The remainder of the document provides guidelines to assist in caring for fishes in laboratory facilities, followed by guidelines to help in the development and review of experimental protocols. An overview of the CCAC *guidelines on: the care and use of fish in research, teaching and testing* is provided through a summary of the guidelines listed in this document prior to the beginning of the main text.

The refinement of animal care and use guidelines is a continuous process. These guidelines are intended to provide assistance in the implementation of best practices, and should not be viewed as regulations. Where regulatory requirements are involved or where it is absolutely imperative to adhere to a particular guideline, the term *must* has been used.

B. INTRODUCTION

The greatest challenge in providing *guidelines on: the care and use of fish* is the wide variety of fishes used in Canada and the diversity of their habits, behavior, life history, and environmental and husbandry requirements. In addition, the scientific information required to define the preferred conditions for fish well-being is limited. While considerable research has been conducted on culture strategies and environmental and water quality requirements, such studies have generally been aimed at determining conditions that optimize production in aquaculture systems, rather than improving the welfare of fishes, and have not usually addressed the difference between *tolerance* and *preference* (Fisher, 2000).

An important consideration in these guidelines is the naturally high mortality rates of juveniles in species whose ecological strategies include the generation of large numbers of progeny to ensure adequate survival in the wild. In addition, many experimental populations of species with usually high survival contain individuals that will not thrive to adulthood even under the best environmental conditions. In some situations, a population-based (or a group of study fish) approach to well-being may be appropriate, but individuals that are not likely to thrive should be euthanized as soon as they are identified.

Another consideration for these guidelines is the general acceptance by the public of the current killing methods used in harvesting wild fishes or in recreational angling. In general, the public appears to be willing to accept these killing methods for food production but not when fishes are used for research. These guidelines accept that for research, teaching, and testing use of any animal, including fishes, more emphasis will be placed on individual well-being than is generally accepted for the commercial harvesting or production of animals for food. It is recognized, however, that in some instances investigators may obtain fishes from people involved in commercial or recreational harvesting and have little influence over the capture methods.

These guidelines apply to fishes held in facilities for research, teaching and testing, as well as to fishes that are studied in their natural habitats.

1. Definition of Fish

For the purpose of these guidelines, fishes are defined as all bony and cartilaginous fish genera (classes Chondrichthyes [cartilaginous fishes], Agnatha, and Osteichthyes [bony fishes]). Fish eggs, embryos or larvae that have not developed beyond exclusive reliance on their own yolk nutrients are not covered by these guidelines. Similarly, invertebrates (except cephalopods) are not covered under the CCAC system of surveillance, but institutions are encouraged to foster respect for these animals by ensuring that holding facilities and levels of husbandry meet standards equivalent to those used for fishes.

2. Rationale for Guidelines on the Care and Use of Fish

The use of fishes as experimental subjects has increased substantially over the past two decades. This increase in use is a result of the rapid development of the aquaculture industry, requirements for testing involving fishes as indicators of environmental change, and the use of fishes as a replacement for mammals in biomedical, pharmacological and genetic research (DeTolla *et al.*, 1995; Fabacher & Little, 2000). The trend toward the use of fishes as a replacement for studies that would previously have used mammals as experimental subjects is not discouraged. However, it must also be recognized that fishes have the capacity to perceive noxious stimuli. Noxious stimuli are those stimuli that are damaging or potentially damaging to normal tissue (e.g., mechanical pressure, extremes of temperature and corrosive chemicals). Whether or not fishes have the capacity to experience any of the adverse states usually associated with pain in mammals is subject to a great deal of debate in the scientific literature (FAWC, 1996; FSBI, 2002; Rose, 2002; Braithwaite & Huntingford, 2004). Nonetheless, fishes are capable of behavioral,

physiological and hormonal responses to stressors (including noxious stimuli) which can be detrimental to their well-being. These CCAC guidelines both support the leadership role that Canadians play in fish research, and ensure that the welfare of fishes is carefully considered during the use of fishes for research, teaching and testing, recognizing that better welfare will result in better science.

3. Ethical Overview

Guideline 1:

Fishes used in research, teaching and testing must be treated with the respect accorded to other vertebrate species.

The CCAC's surveillance system for animals used in research, teaching and testing is based on the principles of humane science, i.e. the Three Rs of Russell and Burch (Russell & Burch, 1959) - Reduction, Replacement and Refinement. For the CCAC, these principles are laid out in its *policy statement on: ethics of animal investigation* (CCAC, 1989). The *ethics of animal investigation* applies to all species covered by the CCAC system, i.e. all vertebrates and cephalopods.

In addition, the CCAC system takes a "moral stewardship" approach to the use of animals in science as explained in the CCAC Experimental Animal User Training Core Topics - Module 2, Ethics in Animal Experimentation (http://www.ccac.ca/en/CCAC_Programs/ETCC/Module02/toc.html).
The first guideline statement in the CCAC *guidelines on: institutional animal user training* (CCAC, 1999a) states, "Institutions must strive through their training programs to sustain an institutional culture of respect for animal life".

3.1 Principles of the Three Rs

According to the CCAC *policy statement on: ethics of animal investigation* (CCAC, 1989), it is the responsibility of the local animal care committee (ACC) to ensure that fishes are used only if the investigator's best efforts to find a non-animal model have failed.

As for any other species covered by the CCAC system, investigators using fishes are required to use the most humane methods on the smallest number of animals necessary to obtain valid information. This requires the use of a sound research strategy, including: identification of key experiments that determine whether a particular line of enquiry is worth pursuing; use of pilot studies; staging of *in vitro* to *in vivo* experiments where possible; and implementation of staged increase in test stimuli where possible (Balls *et al*., 1995). The numbers and species of animals required depend on the questions to be explored. Field studies, aquaculture studies and laboratory studies require different statistical designs; field studies and aquaculture production typically require the use of larger numbers of animals. The life stage of the fishes used in each study will also affect the numbers of animals needed. Studies of early life stages typically require large numbers of individuals. In all cases, studies should be designed to use the fewest animals necessary. Heffner *et al.* (1996) and Festing *et al.* (2002) provide discussions on the appropriate treatment of samples and experimental units. Investigators are encouraged to consult with a statistician to develop study designs that have the appropriate statistical power to accomplish the research objectives (Nickum *et al.*, 2004).

The CCAC *policy statement on: ethics of animal investigation* (CCAC, 1989) also requires adherence to the following principles:

- animals must be maintained in a manner that provides for their optimal health and well-being, consistent with the demands imposed by the experimental protocol;
- animals must not be subjected to pain and/or distress that is avoidable and that is not required by the nature of the relevent protocol;
- expert opinion must attest to the potential value of studies with all animals, including fishes (e.g., scientific merit for research, see CCAC *policy statement on: the importance of independent scientific merit of animal based research projects* [CCAC, 2000a]; pedagogical value for teaching; and the appropriateness of the method to provide data for testing according to current regulatory requirements);
- if pain or distress is a justified component of