DESIGNING A FISH ASSESSMENT PROGRAM FOR HIGH PESTICIDE USE AGRICULTURAL AREAS IN SRI LANKA

by

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ABSTRACT

The increased use of limited land resources in tropical countries used for agricultural activities requires development of ecosystem-based assessment tools to ascertain system sensitivities to protect those ecosystems from impacts. A highly fragile agricultural ecosystem in the upper Mahaweli River basin was selected to address the concepts of "multiple stressors" and "cumulative effects" in the Sri Lankan context. The agricultural catchment exhibited extensive exploitation, which prevented the location of reference sites within the catchment. Furthermore, an interpretation of data from an alternate forested catchment was challenged by the presence of cryptic fish species and shifted reproductive development. The community composition in agriculturally-exploited areas revealed loss of prominent species and/or species groups relative to the forested catchments. Seasonal reproductive patterns of three fish species (*Garra ceylonensis*, *Devario malabaricus* and *Rasbora daniconius*; Teloestei, Cyprinidae) were monitored from 2009 to 2011. The following characteristics of *G. ceylonensis* were most suitable for assessment: numerical abundance, wide distribution, single spawner, lower trophic food preference, and peak reproductive exposure to stressors. The response to agricultural stressors by *G. ceylonensis* primarily followed a eutrophic gradient, such as that this species exhibited increased density, larger body size of individuals, and increased male and female gonad size in response to increasing agricultural land use. In the middle elevations of the catchment, there were sub-optimum gonad investment and fecundity of females suggesting that fish are exposed to chemical stressors, and/or physical stressors. Results of residue analyses of organophosphate (OP) and N-methyl carbamate (NMC) pesticides in water and sediments were related to acetylcholinesterase enzyme activity in three stream-dwelling fish species; total OPs and NMCs and fish responses were higher during drier periods relative to the wet season. The

overall objective of this dissertation research was to integrate current understanding on fish biology and the effects of possible agricultural stressors by linking theoretical and observational information, identifying data gaps, and recommending an overall approach to population risk assessment related to non-point, multiple stressor impacts from agriculture.

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> Let me quote Skinner (1953); "Science is first of all a set of attitudes. It is a disposition to deal with the facts rather than with what someone has said about them. Science rejects even its own authorities when they interfere with the observation of nature. Science is a willingness to accept facts even when they are opposed to wishes. The opposite of wishful thinking is intellectual honesty — an extremely important possession of the successful scientist. Scientists have simply found that being honest — with oneself as much as with others — is essential to progress. Experiments do not always come out as one expects, but the facts must stand and the expectations fall. The subject matter, not the scientist, knows best". ... B.F. Skinner (1904–1990).

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CHAPTER 1

1 General Introduction

1.1 Overview

Agriculture is a non-point source of stress to aquatic ecosystems. Pethiyagoda (1991, 1994, 2006) listed several potential threats to Sri Lanka's freshwater fishes, including degradation of wet zone forests and swamps, pesticides, alien invasive species, exploitation, and hydrological alterations. Surprisingly, only a few studies have been conducted on fish in freshwater ecosystems affected by agriculture and pesticides in Sri Lanka (Wijeyaratne and Pathiratne 2006; Jayasundara and Pathiratne 2008). Most of the previous assessments were primarily conducted for academic interests rather than for environmental assessment or long-term (status and trends) monitoring purposes. Pesticide toxicity to local fish species has been investigated (e.g. De Mel and Pathiratne 2005; Chandrasekera and Pathiratne 2007), though studies were confined to laboratory verifications. In the majority of instances the modulation of acetylcholinesterase (AChE) activity has been the single tool (e.g. biomarker) used to assess the response of fish to contaminant stress.

The impacts of some of the most common pesticides used in Sri Lanka on fish health have been studied using *Oreochromis mossambicus* (fry fingerlings of tilapia) under laboratory conditions (Shafiei and Costa 1990; Pathiratne and Ranasinghe 1992; Pathiratne and Athauda 1998; Pathiratne and George 1998; Pathiratne 1999; Chandrasekara and Pathiratne 2005). Most toxicity studies have focused on exotic fish species, such as *O. mossambicus*, probably because of their abundance and ease to keep in captivity. A recent study by Wijeyaratne and Pathiratne (2006) examined *Rasbora caverii*, an indigenous fish species in Sri Lanka, for impacts near rice fields exposed to paraquat, fenthion, and phenthoate. *Rasbora caverii* was shown to be markedly more sensitive to brain AChE depression over *O. mossambicus*, emphasizing the importance of

obtaining information on native species. Moreover, predictions on the risks of pesticides and associated agricultural inputs are difficult to extrapolate from simple laboratory studies. Population level assessment of agricultural impact using indigenous fish species is of high relevance to the public, but the required methods have largely been limited to temperate environments, and extrapolation of data to tropical conditions is rather fuzzy (De Silva et al. 2009; De Silva and van Gestel 2009). Therefore, there is a clear need to undertake a more comprehensive assessment of the potential impacts of agricultural practices on fish populations in Sri Lankan river systems.

Agricultural ecosystems in developing countries have been invariably impacted by high pesticide inputs and ecosystem alterations. Current research in these countries is usually limited to laboratory verifications and research on broader relevance in ecological settings is scarce.

1.2 Sri Lankan rivers

Sri Lanka (6-10°N, 80-82°E) is part of the Indian sub-continent, and although it is small in area (65,621 km^2), it is an ecologically diverse landmass, that is drained by 103 rivers (Silva 1993). The central cross section of the island is characterized by a three-peneplane altitudinal model (Vitanage 1970), which divides prominent slope breaks into coastal lowlands (up to 270 m and 0 to 15 degrees slope), uplands (270 to 1,060 m altitude and 10 to 35 degrees slope), and highlands with well-defined high plains and plateau (910–2,424 m). Most of these rivers originate from the central mountain region and flow through low altitude coastal areas to the sea. The river basins cover over 90% of the island, and rivers constitute about 6.4% of the total land area (Somasekaram 1997). Many of the lowland reaches of the major rivers were dammed to make man-made lakes (reservoirs).

On the basis of the seasonal distribution of rainfall, Sri Lanka is divided into wet and dry zones (Domroes 1979). The wet zone receives rainfall from the southwest and northeast

monsoons, which provide an average annual rainfall of 2,400 mm. The wet zone mountains act as a buffer, and feed the streams throughout the year – therefore, wet zone streams are generally perennial. The dry zone covers about ¾ of the land situated below 900 m above mean sea level (MSL), with an annual rainfall of 1,450 mm on average. The northeast monsoon feeds the rivers in the dry zone from December to April, but from May to November (i.e. dry season) streams go dry (completely or partially). In this sense, the monsoon is a determinant factor for aquatic life in the running waters in Sri Lanka. Most freshwater fish species breed during or after the monsoon floods, when plenty of water is available for the young to spread over a wide area, where crowding is less and planktonic food supply is plenty (Fernando and Weerawardhane 2002).

1.3 Agricultural impacts

Impacts on agriculture-based ecosystems are primarily diffuse and variable (Cooper 1993); therefore the temporal and spatial patterns and quantities of contaminants (e.g. sediment, pesticides, and nutrients) are difficult to predict (Gray and Munkittrick 2005). There are various potential sources of stressors on fish such as physical disturbance (suspended solids, temperature), organic enrichment (domestic and urban discharges), toxic materials (inorganic and organic pollutants, heavy metals, pesticides), and development activities (channel modification, construction of dams, and land development) (Scanlon et al. 2007).

In monsoon tropics, agriculture is primarily dependent upon the rainfall pattern. Tropical agriculture is heavily dependent on pesticides for pest and disease control (Ecobichon 2001), and warm climate and associated ecological factors may augment the severity of these stressors (Pimentel et al. 1991). In fact, fungal diseases are aggravated in the rain fed ("wet" season) upland crops (e.g. potato, tomato, and chili pepper), in particular late blight (*Phytophthora infestans*) due to damp weather and lush crop growth. In contrast, irrigated ("dry" season) crops are attacked by plant sucking insects more intensively than "wet" season crops (due to "wash out

effect"), favouring greater application of insecticides. These are the seasonal characteristics that compound the risk of pesticide contamination in tropical agricultural areas.

Often rainfall induced contaminant loadings and fluxes contribute to water pollution in agricultural areas (Huber et al. 2000; Bergamaschi et al. 2001), affecting populations (Gray et al. 2005; Gray and Munkittrick 2005) and community composition of aquatic invertebrates (Liess and von der Ohe 2005; Schulz 2004), and vertebrates including fish (Gray et al. 2005). Pesticide transport studies in agricultural catchments have demonstrated that the majority of pesticides applied on lands enter *via* surface water runoff (Wauchope 1978; Van der Werf 1996), whereas losses *via* subsurface flow (Logan et al. 1994) or spray drift (Kreuger 1998), are less relevant. Surface and ground water chemistry are affected by monsoonal rainfall, and modeling of NO_3^- pollution in streams has been strongly related to land use/cover and flow patterns (Dayawansa 2003). Ranasinghe et al. (2008) revealed excess aluminium (Al) and lead (Pb) concentrations in sediments close to agricultural and urban areas, suggesting impacts of anthropogenic activities. Bandara et al. (2011) estimated cadmium (Cd) in several locations in streams running through agricultural areas and reported mean concentrations in a range of 10.1 to 20 $\mu g\ l^{-1}$ of water, purportedly by use of triple-super phosphate fertilizers with high levels of Cd contaminant. Soil erosion is another characteristic feature of intensive agricultural catchments caused by water runoff due to deforestation, intensive agricultural practices on fields with steep slopes (>35°), and poor handling of water resources (Gunathilake and Gopalakrishnan 1999; Mungai et al. 2004). A post-project environmental evaluation report (Tolisano et al. 1993) showed cases of major agricultural system impairments including (a) chemical inputs, which degraded local surface and ground water quality; (b) inappropriate cropping patterns for the soil and microclimatic conditions of the site, resulting in reduced productivity and increased reliance on environmentally inappropriate agricultural practices; and (c) poor water management practices, which have increased the threat of soil salinization and on-site soil losses.

Located in the tropical belt, Sri Lanka is endowed with a clear pattern of monsoonal climates favouring seasonal shift in agriculture. The small-scale farming plots and two to three cropping sequences per year have resulted in heavy use of agrochemicals, including pesticides (Dharmakeerthi et al. 2011). More importantly, the contemporary agricultural practices have moved away from traditional subsistence agriculture, and expectations of high yield with relatively low tolerance to pest losses have exacerbated the pesticide use. Nearly 40% of Sri Lankan agricultural systems are domestically-oriented, predominantly in small-scale farming units, with rice paddies as the main crop and other subsidiary crops, such as fruits and vegetables (Census of Agriculture 2008). These characteristics are common in farming systems in the developing world (Berg 2001; Ntow et al. 2008).

Pesticide use in Sri Lanka has increased 110-fold from the 1970s up until 1995 without a major change in agricultural land use (Wilson 1999). The current pesticide consumption rate in Sri Lanka (2008 statistics) has reached more than 6,000 tons of formulated products with an average annual use increment of 7% since 1995 (Office of the Registrar of Pesticides, Sri Lanka). These values suggest that, in addition to the net requirement for crop protection, pesticides have been over-exploited in quantities higher than recommended (Jayathilake and Bandara 1989; Aponso et al. 2003). The small-scale farming plots and two to three cropping sequences per year have shown heaviest use of pesticides, exceeding 23 kg ha^{-1} $year^{-1}$ a.i. (Sumith and Munkittrick 2011a). A considerable proportion of pesticides used in agriculture to control undesirable insects are organophosphates (OPs) and N-methyl carbamates (NMCs). These classes of insecticides comprise over 88% of the total insecticide market. Besides, there are over 116 active ingredients registered for use in agricultural applications by 2010 (Office of the Registrar of Pesticides, Sri Lanka).

1.3.1 Agricultural impact on fish communities

Fish communities in streams running through agricultural catchments may be strongly impacted by past catchment land use and possible previous channelization (Harding et al. 1998). The degraded habitats in agricultural sites, as well as the degraded fish communities, may reflect previous modification. In a study of current and historical land use of streams in forested and agricultural catchments, Harding et al. (1998) found that land use in the 1950s, and not current land use, best explained current fish diversity in streams. The species composition of streams with forested riparian that had been surrounded by agriculture in the 1950's was more similar to those of agricultural than other forested streams. Hydrological regimes have also been shown to strongly impact the community structure of fish (Poff and Allan 1995). The effectiveness of riparian habitat in ameliorating water quality and biotic community degradation is clearly shown from the results of previous studies (Martin-Smith and Laird 1998; Lyons et al. 2000), though their effectiveness may depend on vegetative composition, width, and proximity to sources of polluted runoff (Johnson et al. 1997). Crail et al. (2011) showed that herbaceous riparian vegetation in lower order streams in agricultural catchments favour significant increases in fish assemblage measurements of richness, diversity, and feeding guilds. Interestingly, heterogeneity in available habitats has created favourable niches for native fish species with few dominant yet tolerant species, more native species, and only a very small proportion of the exotic fish species.

1.4 Population level performance measures

The assessment of environmental stressors and their biological impacts are often challenging because of the complex nature of ecological systems, and the many biotic and abiotic factors, which can influence or modify responses of biological systems to stressors (McCarty and Munkittrick 1996). In order to have a reliable stressor-response assessment, Karr (1993) proposed the use of multiple endpoints, rather than using a single response variable.

Therefore, two levels of assessment are fundamentally employed (Adams et al. 2005); (1) responses at the lower levels of biological organization (represented by high response sensitivity and specificity), and (2) some responses at the higher levels of biological responses of a fish species to several types of stressors at the population level can be predicted by certain performance measures, such as age structure, energy storage, and energy expenditure. Figure 1-2 summarizes the proposed patterns of fish population responses to environmental stressors.

Survival, growth and reproduction are essential components in maintaining viable fish populations. The performance of fish populations can be limited by several factors, including the relative abundance of food. For obvious reasons, adequate food supply would increase the somatic and reproductive growth (Gibbons and Munkittrick 1994), and the excess energy, which is not immediately required, is stored in tissues. Measurements of energy utilization include gonad size, the ratio of gonad weight to body weight (gonadosomatic index; GSI), length, weight, size-at-age, fecundity, and egg size. Eutrophication can cause an increased food supply for fish associated with increased primary production. A typical eutrophication response would result in increased energy use and storage (Gibbons and Munkittrick 1994). In the absence of density-dependent limits, the increased reproductive investment would result in a larger number of younger fish, shifting the age distribution of the population towards younger fish (Gibbons and Munkittrick 1994), though these patterns are not definitive (Finley 2008). For example, a large reproductive potential or high fecundity doesn't necessarily translate into a large young-of-the-year recruitment and overall population success because the effects of density-dependent (less competition) compensation mechanisms may also influence success of fish populations.

Some of the indicators that respond to limitations in food availability include reduced condition (i.e. thinner fish) and smaller livers (Munkittrick and Dixon 1989; Gibbons and Munkittrick 1994). The common measurements of energy storage include condition factor, the ratio of body weight to length, liver size, the ratio of liver weight to body weight (liversomatic

index; LSI), and tissue lipids. Nevertheless, the use of condition factor (K) and the LSI have been subjected to uncertainty in assessing pollutant effects in fish (de la Torre et al. 2005; Wijeyarathne and Pathirathne 2006). Williams and Latropoulos (2002) found that increased LSI might be an adaptive strategy associated with hyperplasia of hepatic cells in response to increased functional demand. Changes in the quantity of fat and glycogen stored in the liver have been shown to be supplanted by water without significant changes in the liver size. Therefore, changes in liver size in relation to pollution effects should be interpreted with caution.

Indicators of relative organ size such as LSI and GSI have been widely used for determination of the balance in energy utilization. Litaay and De Silva (2003) have shown inverse trends in between GSI and LSI. In that situation, the discrepancy has been due to using energy stores in the liver to meet the energy demand for gonad growth as the spawning season progresses. The reverse trend is true after spawning. In addition, synchronous and asynchronous spawners are expected to be different in their energy investment in gonadal growth, such that liver storage (measured in terms of LSI) in synchronous spawners could be inversely proportionate with gonadal growth (measured in terms of GSI) (Tyler and Sumpter 1996).

1.4.1 Basic challenges in the determination of fish reproduction

Studies on reproductive biology of tropical freshwater fishes are very limited (Lowe-McConnell 1987). Reproductive strategies in freshwater fish species are as diverse as the species composition (Helfman et al. 1997). Although the Cyprinidae is one of the largest families of fishes, gonadal development has been studied in only a very limited number of cyprinid species (see review by Tyler and Sumpter 1996).

Reproduction of many fish species appears to follow definite seasons. Unlike temperate fishes, where reproduction often occurs during definite seasons, most equatorial fishes breed all year round. Temperate fishes receive cues for gonadal recrudescence from temperature and/or photoperiod. In contrast, many of the tropical freshwater fishes exhibit rainfall-induced

synchronization in their spawning activities (Reynolds 1974). Whatever the reproductive seasonality that a fish species may undergo, their life history strategy usually dictates that the young survive because of optimal access or timing to food availability (Coward and Bromage 2000).

The GSI is a useful indicator to identify periods when the majority of fish develop mature ovaries. A GSI <1% can usually be used to classify immature fish (stage I) or individuals with early endogenous vitellogenesis stages (stage II) of egg development (Environment Canada 2010). For example, Brewer et al. (2008) studied small-bodied riverine fish species during spawning periods and showed that the relevance of using the GSI would be increased when multiple spawning fishes were studied. In such case, the GSI was indicative of broad reproductive periods or reproductive peaks in fishes, rather than the time of spawning (i.e. the release of mature eggs). In contrast, Rinchard and Kestemont (1996) showed that GSI curve would be the best indicator of the time of fish spawning in single spawners, rather than multiple spawning fish. In the broader category, single spawners (i.e. spawn once per year) show group-synchronous oocyte development, and have a short spawning period. Similarly, multiple spawners spawn multiple clutches of eggs per reproductive period and have a prolonged reproductive period (Barrett and Munkittrick 2010). By studying the reproductive biology of three temperate cyprinid fishes, Rinchard and Kestemont (1996) showed that multiple spawning fishes (the bleak *Alburnus alburnus* and the white bream *Blicca bjoerkna*) have the lowest GSIs because of their protracted and batch spawning, while single spawners have the highest GSI since their gonadal recruitment occurs only once in a season. However, the reproductive condition of fish can be conveniently assessed if a set or suite of parameters (viz. macroscopic aspects of ovaries, GSI, examination of oocytes, and blood steroid levels) are evaluated together (Stéquert et al. 2001).

1.5 Fish tissue acetylcholinesterase activity

Acetylcholine (ACh) is a neurotransmitter that is secreted from pre-synaptic nerve terminals in mixed muscarinic and nicotinic cholinergic synapses in the central and neuromuscular junctions (Richetti et al. 2011). After release, ACh is rapidly removed from the synaptic cleft by acetylcholinesterase (AChE, EC 3.1.1.7), which belongs to the family of serine hydrolase, and hydrolyzes acetylcholine into choline and acetate (Chatonnet and Lockridge 1989). Organophosphates (OP) and N-methyl carbamates (NMC) bind to the AChE active site and serve as surrogate substrates for the enzyme. The covalently reacted phosphorylation or carboxylation within the active sites, respectively, prevents neurotransmitter binding and hydrolysis. The major distinction between the two classes of insecticides is the duration of AChE inhibition. Whereas, OP-induced inhibition is effectively irreversible, inhibition by NMC is reversible, which leads to a faster rate of recovery from enzyme inhibition (Scholz et al. 2006).

The inhibition of acetylcholinesterase (AChE) activity has been instrumental in biomonitoring of OP and NMC intoxication in fish (Fulton and Key 2001; Wijeyaratne and Pathiratne 1996; Guimarães et al. 2007; Jayasundara and Pathiratne 2008). The AChE enzyme activity in the brain, the skeletal muscle, eye, and the liver are common assessment sites in fish. However, the inhibition of brain AChE would be more rational for direct pesticide toxicity than that of muscle AChE (Carr et al. 1997). In addition to that higher measurable AChE activity in fish brain, brains of cyprinids (Chuiko 2000) are the central organ for neurobehavioural functions from neuromuscular activation to cognitive function, such as locomotion, food searching, and response to environmental perturbations. The liver contains an alternate form of cholinesterase, i.e. BuChE (butyrylcholinesterase), which is less useful for AChE-mediated toxicity assessments (Fulton and Key 2001).

Acetylcholinesterase activity has been examined in response to exposure to single

individual compounds in the majority of studies that have been conducted on its activity (Guimarães et al. 2007; Chandrasekare and Pathiratne 2007). AChE activity has also been correlated with dominant AChE-inhibitory compounds in non-point contamination situations (Forget et al. 2003). Contemporary choices of pesticides for farmers in Sri Lanka encompass relatively less persistent properties that degrade quickly under hot tropical climatic conditions and the residue from most commonly used pesticides may not be detectable in water (Aponso et al. 2003). These considerations suggest that the potential for impacts at the catchment level or from season-to-season can be low (van der Werf 1996), but ecotoxicological assessments using a sensitive "biomarker" such as AChE for neurotoxic pesticide exposure is highly appropriate.

1.6 Study site

The Mahaweli *Ganga* is the largest of 103 rivers in Sri Lanka, and is the only perennial river crossing the dry zone of the north-central area. These perennial rivers are called *Ganga*, while streams are called *Oya* in native Sinhalese or *Aaru* in Tamil language. In Sri Lanka, 92 rivers are called *Oya* or *Aaru*, only 11 are *Gangas* (Weliange 2007). The Uma-oya is one of the major tributaries of the Mahaweli River, and is probably the largest in the upper catchment, and is the main focal area for the study.

1.6.1 Land use pattern in the Uma-oya catchment

The Uma-oya drainage area in the Upper Mahaweli Catchment (UMC) geomorphologically consists of mountain slopes, narrow ridge slopes, low rounded hills of moderate relief, foothills and foot-slopes, dissected plateau, undulating valley plains and valley flats. Uma-oya is a main tributary of river Mahaweli, and is the only main stream draining into Rantembe reservoir, the last of the chain of reservoirs constructed under the Accelerated Mahaweli Development Project (AMDP). The mean annual rainfall is around 2,500 to 3,000 mm in the wet zone region, and it is around 2,000 to 2,500 mm in the intermediate region of

catchment. Because of the elevation, the highest daily average maximum temperature of 21.5°C occurs in April, and the lowest mean daily minimum of 10.3°C in January in Nuwara Eliya area. The average annual temperature in Welimada area is 27°C (Dayawansa 2003).

Environmental conditions and ecological integrity have been degraded with the advent of indiscriminate land use changes in the Uma-oya catchment over the years. Hewawasam (2010) reported tea lands declined by 60% from 1956 to 1991, while the extent of non-tea crops (mainly vegetables) increased by 300% in the UMC. With the gaining popularity, the Uma-oya catchment evolved into Sri Lanka's major "vegetable hub", with vegetables being the most important agricultural crop in lowland and highland fields. The average farm size is quite small at 0.28 ha (± 0.25 SD) and 0.26 ha (± 0.16 SD), in lowland and highland fields, respectively. The farming systems use the valley bottoms for rice paddy cultivation during the main rainy season, maximizing the land use and food demand. The gross cropping pattern in the catchment is composed of 95% rice paddy during February and May, and 93% potato and bean during July and September in lowlands. More than 68% of highland fields are grown with potatoes and beans during October and January, while lowland fields are still completing their growth cycle in this season. Home gardens with perennial crops have been established adjacent to lowlands and valley bottoms. Unlike in other catchments, hilltops are extensively cleared for cultivation and a small extent of forest plantations of eucalyptus (*Eucalyptus* spp.) and pines (*Pinus* spp.) are present (Sumith and Munkittrick 2011b).

1.6.2 Pesticide transport into the Uma-oya

The main pesticide application periods are the *Yala* season (June to August), and the *Maha* season (October to January); the latter is characterized by frequent rainfall events, raising the possibility of high inputs of waterborne contaminants through runoff. The *Yala* season is characterized by fairly mild and infrequent rainfall events, but there are sufficient rainfall events in the highland tributaries in the upper catchment for water requirements for agriculture.

The irrigable lowland fields in the Uma-oya catchment are prepared for vegetable cultivation during the months of June and July. During successive crop growth periods (July through September), a copious amount of water is used for irrigation; typically, furrows are inundated with water from feeding channels and manual panning (*"Thatiganawa"*) of water onto crops is the most common method of irrigation, though some farmers tend to pump water from adjacent streams onto vegetable plots directly. Then, excess water is allowed to drain into adjacent streams at the end of each event. These releases can be viewed as episodic exposures to agricultural stressors (e.g. pesticides, toxic (heavy) metals, and nutrients), when free and bound pollutants can be re-suspended and transported with the tail-water. Therefore, sediment transport during the land preparation and irrigation events is most often intensive and can be episodic.

1.7 Objectives and hypotheses of dissertation

The Canadian Environmental Effects Monitoring (EEM) program has been used to evaluate the health of waste streams from pulp and paper mills, metal mining fields, and sewage treatment facilities using ecologically relevant fish endpoints for more than a decade (Lowell et al. 2003). Such a program incorporates a broad understanding of effects- and stressor-based assessment methodologies (Gray et al. 2002, 2005; Gray and Munkittrick 2005) along with stressor-impact correlations for point source pollutants (Galloway et al. 2003; Sandström and Neuman 2003; Chiang et al. 2011). These methods have also been used to examine biological effects due to agricultural non-point sources using the slimy sculpin (*Cottus cognatus*) (Gray et al. 2005; Brasfield 2007), although the studies used a gradient design that effectively treated the tributaries as point sources. There are several fish population response patterns (Munkittrick et al. 2000) that would be expected in agricultural ecosystems, the most likely of which would be due to nutrient enrichment or metabolic disruption. The utility of small-bodied cyprinid fishes for EEM programs have well been recognized (Galloway and Munkittrick 2006; Barrett and

Munkittrick 2010), but complete baseline life history information is important for proper study design and data assessments.

Therefore, the overall objective of this research program is to develop a sensitive environmental monitoring program that identifies biological responses, the mechanisms of impact, and the stressors potentially responsible for any impacts on fish populations in agricultural areas in Sri Lanka. The long term objectives of this project are to develop a better understanding of the life history and potential parameters of fish (i.e. reproduction) influenced by agricultural stressors and to determine how sentinel fish species can be utilized to design more environmentally sustainable and sensitive monitoring programs for agricultural impacts, and thus minimize potential environmental impacts to ecological communities.

1.7.1 Statement of the problem

The focus of the dissertation is to design a fish assessment program for high pesticide use agricultural areas in Sri Lanka. To do this it is necessary to understand the potential responses of native fish populations in Sri Lankan rivers to agricultural practices under high pesticide use. The dissertation will focus on a major river system where pesticide use is very high, and will develop a river assessment procedure for looking at the potential impacts on fish, and develop appropriate follow-up studies to provide recommendations for future assessments. The protocol developed should be broadly applicable to agricultural investigations in south and southeast Asia. A conceptual framework for assessing the health of fish residing in streams in agricultural catchments is presented in Figure 1-3.

The specific hypotheses tested will include:

H_0-1: There are no differences in community composition of fish species at high risk agricultural sites and reference locations

H_0-2: There are no differences in performance characteristics in fish species at high risk agricultural sites and reference locations

H_0-3: There are no differences in the AChE responses of fish species at high risk agricultural sites and reference locations

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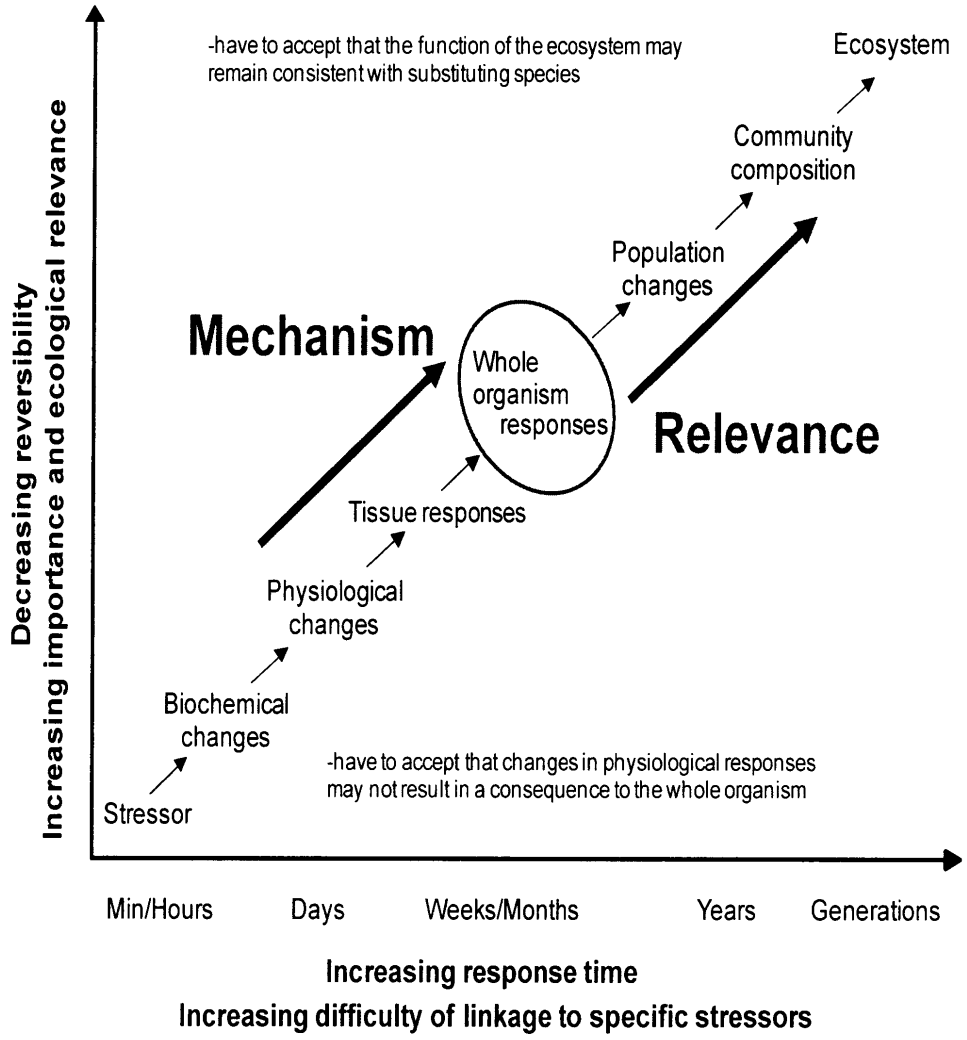

Figure 1-1. Levels of biological organization (= mechanism) and the ecological relevance (= relevance) and time scale response. The difficulty of measurement, response time for changes, and ecological significance increase with increasing biological levels. Responses shown at the left of Figure 1-1 (e.g. biomolecular/ biochemical) are essentially quick-responding (within minutes and hours) and sensitive indicators to environmental stressors, but they are generally characterized by low ecological significance. Responses at the right side of Figure 1-1 (e.g. population and community-level), however, display very little response sensitivity (which can span for years or biological generations), but possess high ecological relevance (modified from Munkittrick and McCarthy 1995).

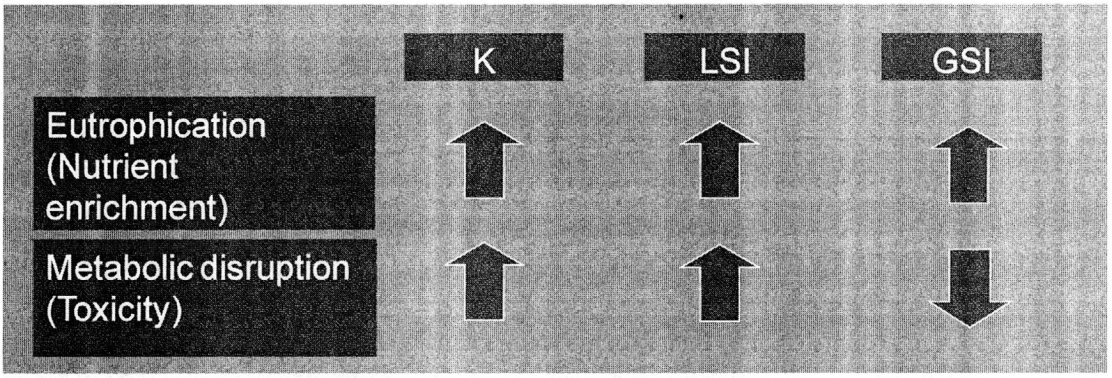

Figure 1-2. Patterns of population responses previously observed in wild fish populations. Gonadosomatic index, GSI (gonad weight/body weight)*100), liversomatic index, LSI (liver weight/body weight)*100) and condition factor, K [(weight/length^3) *100], Increase response↑ Decrease response↓ (modified from Gibbons and Munkittrick 1994).

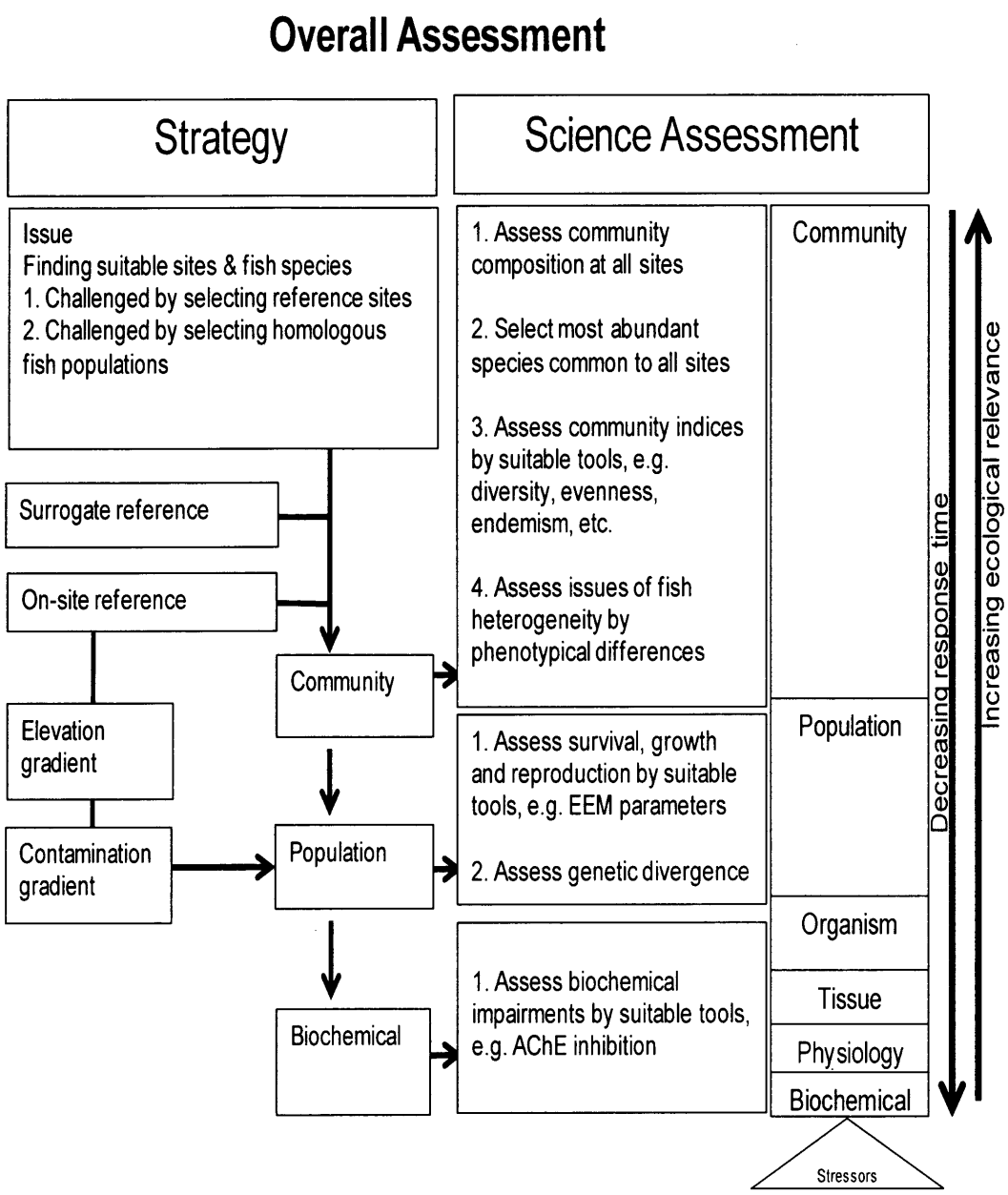

Figure 1-3. Cumulative impact assessment framework used in the Uma-oya agricultural catchment– (which is based on the premise that broader catchment studies can be challenged by selecting reference locations for baseline data and subject organism's heterogeneity (here fish species), and integrating such study components in selecting study sites, fish species and tools for a viable assessment, encompassing levels of impact assessment hierarchy).

CHAPTER 2

2 Study design considerations for assessing the health of fish populations impacted by agriculture in developing countries: a Sri Lankan case study [1]

2.1 Abstract

Studies on the use of indigenous or endemic species for the assessment of agricultural impacts on fish populations are lacking in tropical south and southeast Asia. This chapter describes the process involved in developing an agricultural impacts assessment program focused on river health using recent studies on wild fish in Sri Lanka. The assessment methodology includes the selection of fish species for monitoring, development of a strategy for sample timing, sample size requirements, and selection of appropriate reference site(s). Preliminary fish sampling data from several tributaries of the Uma-oya and the Amban-ganga (Knuckles streams) from the Mahaweli River basin were evaluated using data collected which examined temporal patterns of gonadal recruitment were investigated for three common species: *Garra ceylonensis*, *Devario malabaricus*, and *Rasbora daniconius*. The data on reproductive development were statistically analyzed to evaluate appropriate sample timing and sample size requirements. For this study, we used a cluster gradient design with a range of assessment endpoints, and compared results to least contaminated sites and reference sites in a less contaminated surrogate reference catchment in a national forest reserve. The review and preliminary data provides a template for study design considerations for agricultural impact assessments in south and southeast Asian countries.

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Keywords: Agricultural catchment, *Devario malabaricus*, Exotic fish, *Garra ceylonensis*, Knuckles streams, Mahaweli River basin, Nitrate, Phosphate, *Rasbora daniconius*, Sri Lanka.

2.2 Introduction

Agricultural runoff has been implicated in the destruction and disturbance of the integrity of aquatic flora and fauna worldwide (Dudgeon 1996; Bergamaschi et al. 2001; Gray et al. 2005). A number of agrochemicals have been detected in surface waters and several studies have demonstrated significant negative effects of agrochemicals on aquatic organisms and ecosystems (Jergentz et al. 2004; Thiere and Schulz 2004; Purcell and Giberson 2007). Depending on weather conditions and field slope, up to about 10% of applied pesticides may be transported in runoff from agricultural fields (Wauchope 1978; Schulz 2004). Cumulative non-point source pollutants, including increased turbidity and sedimentation from land use have brought pervasive impacts on resident organisms (Berkman and Rabeni 1987; Olvera-Viascán et al. 1998; Gray et al. 2005). Next to direct impacts, indirect consequences of agricultural activities include altered hydrology, changes in stream temperature (direct effect from altered riparian vegetation), habitat alteration, and impact on food sources and reduced oxygen levels (Bunn et al. 1999; Casatti et al. 2006), which further complicates the situation. These stressors combined can have great impacts on the aquatic ecosystem, for example, the US EPA estimated that routine agricultural activities and non-point sources were responsible for 45% of the nation's river and stream pollution (US EPA 2009).

Although the importance of agricultural activities as a stressor for fish has been recognized in Asia (Hussain 2010), impacts have not been widely studied, and studies are usually restricted to biomarker responses (Shafiei and Costa 1990; Pathiratne and Ranasinghe 1992; Pathiratne and Athauda 1998; Pathiratne 1999; Chandrasekara and Pathiratne 2007; Pathiratne et al. 2009), or impacts of agriculture on fish captures and harvests (Khoa et al. 2005).

There are a few recent Asian studies that have shown fish inhabiting natural freshwater ecosystems may be affected by intentional and unintentional spreading of pesticides (Wijeyaratne and Pathiratne 2006; Jayasundara and Pathiratne 2008). Studies assessing population level responses to agricultural stressors are scarce. Even though there is little research conducted to date, agricultural practices in south Asia place a variety of stresses on aquatic systems (Atapattu and Kodithuwakku 2009), with agricultural activities and associated pesticide use often being blamed for the decline of valued fish populations (De Silva and Kortmulder 1977; Moyle and Senanayake 1984; Pethiyagoda 1994; Amarasinghe et al. 2006; Pethiyagoda 2006). To date, research on the ecological impacts of agriculture on river systems has been hindered in Asia by incomplete life history of biota including fish species (Dudgeon 2000), and increasing attention is being placed on maintaining biodiversity and in-stream ecological needs (Dudgeon 2005; Nguyen and De Silva 2006). In order to protect the biodiversity of streams in agricultural areas, survival, growth, and reproduction of resident fish species should be a priority in bioassessment programs. However, there is generally inadequate baseline knowledge in developing countries on resident biota, or the performance of reference populations on which to base such an assessment. Therefore, highly coordinated research studies focusing on locally abundant fish species are a high priority for developing a baseline and approach for bioassessment.

The Canadian government developed a program for Environmental Effects Monitoring (EEM) that has been applied to assessing fish population health at sites discharging pulp and paper mill effluent since 1992 (Munkittrick et al. 2002; Munkittrick et al. 2010), and metal mines since the early 2000's (Ribey et al. 2002). This approach uses indicators of survival (mean age, age distribution or size distributions), the efficiency of energy use (growth rate and reproductive investment), and energy storage (liver size and fish condition) relative to a reference site(s) to assess anthropogenic impacts (Barrett et al. 2010). The Canadian EEM approach is an adaptive,

iterative sampling framework, with subsequent monitoring rounds based on the magnitude and types of effects seen in earlier assessment(s). The ability to interpret responses varies with the receiving environment (barriers, effluent concentrations, habitat quality), the life history characteristics of the species sampled (mobility, longevity, proximity of spawning areas, etc.), and the characteristics of the stressors (of importance) in the area (uptake pathways, half-lives, interaction of multiple stressors, exposure concentrations). Under most circumstances, the life history characteristics of species do not respond in isolation to the impacts of the stressors, but several endpoints respond together (i.e. a change in growth has consequences for changes in size at maturity, which over a chronic exposure will impact the age distribution). An effects-based EEM approach is under development for sites receiving sewage effluent (Kilgour et al. 2005), has been applied to agricultural impacts (Gray et al. 2005), and has been adopted for use in other countries, including Cuba (Aguilar et al. 2007), and Chile (Chiang et al. 2010).

This chapter presents a rationale for developing a study design for assessing the ecological importance of agricultural impacts on fish in south and southeast Asian river systems, using Sri Lankan rivers as a case study. The approach will rely on a combination of results from preliminary sampling and from limited published literature. The preliminary sampling was used to iteratively develop the study design that considers the selection of suitable fish species, best sampling periods, and sample size requirements using appropriate statistical methods. Munkittrick (1992) proposed five basic considerations for the development of the study design for site-specific assessment of health of fish populations including, (1) choice of monitoring level and strategy, (2) selection of species, (3) timing of sampling, (4) sample size requirements, and (5) selection of appropriate reference site(s). This guidance has been adopted for development of study designs and species selection for the Canadian EEM program (Environment Canada 2010), and this chapter incorporates the Canadian experience, and the challenges within Sri Lankan context.

2.3 Materials and Methods

2.3.1 Study area

Most of Sri Lanka's intensive cropping systems are distributed in the highland plateau above 450 m above mean sea level (MSL). Sri Lanka has a total of 103 rivers that drain radially from a central highland. The Mahaweli River is the longest river (335 km) with the highest discharge in Sri Lanka, and has a basin area of approximately 20% (10,327 km^2) of the total area of the country (Silva 1993). The upper basin covers approximately 25% of the central highland in Sri Lanka with more than five distinct catchments, of which the highly intensive agricultural Uma-oya catchment is the largest at 886 km^2 (Hewawasam et al. 2003; Ranasinghe et al. 2008), with a human population of 1.48 million (2001 census). The Uma-oya is a main tributary of the Mahaweli River and is the main source draining into the Rantembe reservoir; the last of the chain of reservoirs constructed under the Accelerated Mahaweli Development Project (1979–1984) (Hewavisenthi 1997). Mean annual discharge of the Uma-oya (stream) during the hydrologic months from October, 1992 to March, 1993 (40.4 million m^3) was twice the amount compared to the mean discharge for the period from April-September, 1993 (19 million m^3) (Mahaweli Authority of Sri Lanka). Although, locally "oya" is a term for streams that dry up during the dry season (Hewavisenthi 1997), feeder streams of the Uma-oya receive sufficient rainfall throughout the year to maintain a continuous flow. The Uma-oya catchment has a steep topography (mean slopes range from 5 to 30°) with the elevation ranging from 915 to 2,440 m above MSL and a mean annual rainfall ranging from 2,500 to 3,000 mm in the wet zone region, and 2,000 to 2,500 mm in the intermediate region. The average annual temperature in the Uma-oya catchment is 27°C (Dayawansa 2003). The total extent of upper Uma-oya (above the gauging station at Welimada) is 146 km^2, and extends from the towns of Nuwara Eliya to Welimada.

The main two tributaries of upper Uma-oya (Bomburuella-oya and Nanu-oya) confluence about 5 km downstream of Welimada town. The lower region of the upper catchment (Welimada) is comparatively scarcely populated, and the crops cultivated and the farming methods practiced, are different to those of in Nuwara Eliya. In the upper catchment, farmers tend to cultivate three or four times in a year on the same land with vegetables, such as potato, leeks, beet, chili pepper, and carrot that require high inputs of fertilizer (Wijewardena 1999), and intensive use of agrochemicals (Burleigh et al. 1998; Aponso et al. 2003). A 2008–2009 survey of 110 representative farmers in three localities within the Uma-oya catchment revealed that the environmental load of pesticides was as high as 23,434 g ha^{-1} active ingredient (a.i.) (Sumith and Munkittrick 2011), which is >24 times higher than the national average of 970 g ha^{-1} a.i. (FAO 1997). Pesticide application methods are variable and poorly controlled, causing a considerable amount of surplus in the environment, leaving the entire catchment at risk. In the middle catchment, potatoes and rice are cultivated in the same fields in alternative seasons, with a third crop established after potatoes. Land use change assessment in the Uma-oya catchment between years 1956 and 1992 has shown that former tea plantations have been replaced largely by annual vegetable crops (Chandrasekera et al. 2000).

Surface and ground water chemistry have shown to be affected by the monsoonal rainfall where modeling of NO_3^- pollution in streams was strongly related to land use/cover and flow patterns (Dayawansa 2003). Ranasinghe et al. (2008) revealed excess Al and Pb concentrations in sediments close to agricultural and urban areas, suggesting impacts of anthropogenic activities. Intensive human activities in the upper Uma-oya catchment have caused annual sediment yields as measured by suspended river-load fluxes at 130 to 2,100 t km^{-2}. The local rate of soil loss has been estimated as high as 7,000 t km^{-2} $year^{-1}$ in hill slope agricultural lands (Hewawasam et al. 2003), which exceeds the soil loss tolerance value of 5,000 t km^{-2} $year^{-1}$ (Gunathilake and Gopalakrishnan 1999). Nearly all of this sediment accumulates

downstream in the Rantembe reservoir, and 25% of the reservoir was silted within three years of its commission in 1990 (Illukpitiya and Gopalakrishnan 2004). Concerns over the potential agricultural impacts on river ecosystems have increased in recent years.

Twelve sites were selected for the collection of baseline information. The sites were represented by eight locations (i.e. agriculturally impacted sites) of the Uma-oya tributary (streams), and four potential reference locations from the Amban-ganga tributary (Knuckles streams) (Figure 2-1). All sites were within the Mahaweli river basin, representing the Mahaweli ichthyological province, as described by Senanayake (1980) considering its distinct faunal characteristics. The Knuckles range is a rugged hill mass rising to 1,500 m above MSL average height, and the potential reference area is a tropical mountain torrent system in the headwaters of the Amban-ganga. The sampling locations were above the settlement or within the forest catchment in the north-eastern slopes between 250 to 900 m above MSL. The eastern slope of the drainage catchment is influenced by heavy rains during the northeast monsoon from October to January, but during the southwest monsoon, much less rain is received between April and July. The annual rainfall in the wettest area is 2,725 to 4,470 mm. The temperatures range between 25 and 27°C at lower altitudes, and between 18 and 20°C at higher altitudes (Karunaratne and Edirisinghe 2008).

2.3.2 Fish sampling

Fish species were sampled during low-flow months of February, July, September (2009), and January (2010) from various sites in the Uma-oya. Sampling during the northeast monsoon season occurred in January and February sampling months, while samples during the July and September months represented the southwest monsoon season. Preliminary sampling in February 2009 suggested the need for additional reference sites, resulting in sampling sites being added in the Knuckles region in January (2010). A backpack electrofisher (EFKO equipment with Honda aggregate) was used in February and July sampling while composite net sampling

employing three methods of netting (i.e. one cast net, two dip nets, and one drag net of 10 mm stretched mesh size), were used in other months. The electrofishing and net samplings were standardized as far as possible between sites by means of number of passes, time between consecutive passes, and unit area. The streams were very heterogeneous environments, where fish exhibit patchy distributions over spatial and temporal gradients. Stream sections selected included a minimum of two riffles and mini pools. To calculate fish species per unit area (relative abundance), total numbers were divided by the surface area (m^2) of each study reach. For each sampling site, surface area was estimated by multiplying the length of the reach by the mean wetted-width, based on measurements taken at 5-m intervals. For all capture methods in all events, we used the same reach length with three passes that covered the entire reach. We calculated the efficiency of the different capture methods to select methods for subsequent studies.

All fish specimens were taxonomically identified using identification keys (Pethiyagoda 1991; Silva et al. 2008). All specimens of the genus *Garra* were identified as *Garra ceylonensis*, and the genus *Devario* were identified as *Devario malabaricus*, due to subtle or cryptic phenotypical differences of sympatric *G. phillipsi* and *D. aequipinnatus* that can be present in some of the sampling areas. Only adult specimens of total length above 7 cm (of *G. ceylonensis*), 6 cm (of *D. malabaricus*) and 8 cm (of *R. daniconius*) from both sexes were selected for further studies. Fish were anaesthetized by eugenol, and preserved in buffered formalin (10%) immediately after capture for later analysis. Voucher specimens were measured for total length (L) (±0.1 cm) and total weight (W) (±0.001 g) and dissected for gonad weight (±0.001 g) and calculation of gonadosomatic index [GSI=100*(gonad weight/body weight)].

2.3.3 Statistical analysis

For this assessment of agricultural impacts, a rationale was developed for hypothesis testing based on a population-based approach. Our hypothesis was formulated on the assumption

that agricultural activities impact adjacent stream fish species by runoff influenced suspended sediments and associated nutrient and pesticide inputs. Accordingly, the alternate hypothesis (H_1) was that there are measureable "effect(s)" in fish populations to measureable changes in water quality in the Uma-oya. The null hypothesis (H_0) dictates that there are no measureable "effect(s)" in fish populations to measureable changes in water quality. Components of this study were largely descriptive in nature to assess relative fish abundance, to select the most appropriate sentinel species, and to develop a study design for assessment. We used a combination of data collections from twelve sites during four sampling periods and data from previously published studies. Some statistical comparisons were conducted between seasons to develop an understanding of the reproductive cycle in order to select the best sampling times (the null hypothesis for these comparisons would be no statistical difference between sampling seasons). Linear regression analyses from log-transformed data were used to examine the relationships of gonad weight and body weight (Barrett et al. 2010). Monthly GSIs (reported as mean ± SD) were tested using 1–way ANOVA, and similarities in GSI were based on a significant level of $p \leq 0.05$ between the mean GSI of any given month, using the Tukey's multiple comparison test. Basic descriptive statistics on primary data were obtained using Minitab® version 15. Sample size calculations were performed using Power Sample Size Calculation version 3.0.7 (www.biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize). The critical effect size for a priori sample size calculations was set at 25% change in gonad size (Munkittrick et al. 2009), using $\alpha = 0.05$ or 0.10 and $\beta = 0.05$, 0.10, or 0.20 to evaluate the impacts of study design on sample size requirements. Size at maturity was estimated from visual examination of gonad size *versus* body size scatter plots with a regression drawn down through reproductively active fish to intersect the x axis.

2.4 Results and Discussion

2.4.1 Selection of fish species

The initial step in study design involved evaluating the relative abundance of species and the life history characteristics of the more abundant species in order to optimize the sampling design. Sampling across all sites documented 13 species in the Uma-oya (agriculturally impacted locations), and 15 species in the Knuckles streams (reference location), with a total of 28 species being captured across all sites (Table 2-1). A reduced number of fish species was observed with increasing altitude in both catchments. The highest sampling locations in the Uma-oya (Perawella N06°55'25.9" E80°51'49.4"; 1,187 m above MSL) and the Knuckles streams (upper Theligam-oya N07°29'49.8" E80°44'57.8"; 807 m above MSL) were inhabited by only two fish species. In this study, 46% of the species collected in the Uma-oya were cyprinids, while 67% were cyprinids in the Knuckles streams. Zoo-geographically, cyprinids are most diverse in south and southeast Asia (Helfman et al. 1997), with highest species diversity in India, Bangladesh, Myanmar (Spence et al. 2008), Thailand (Beamish et al. 2008), and Sri Lanka (Pethiyagoda 1994).

Of the species examined, three cyprinids were widely distributed in excess of 80% of the total abundance in nine out of twelve sites. In the Uma-oya Ceylon stone sucker (*Garra ceylonensis*) were most abundant at seven sites and giant danio (*Devario malabaricus*) and striped rasbora (*Rasbora daniconius*) were both present in five out of eight sites (Figure 2-2). In the Knuckles streams, *G. ceylonensis* and *D. malabaricus* were present in all four sites, and *R. daniconius* was found in two out of four sites. These three genera are well represented in continental Asia (Froese and Pauly 2010) and the Indian subcontinent (Bhat 2004). There seemed to be small-scale competition between *R. daniconius* and *D. malabaricus* as they were seldom found in high numbers at the same site. The percent abundance of the two species was negatively correlated (Pearson's correlation -0.490, $p = 0.053$) in all sites ($n = 9$) where co-

occurrence was observed (data not shown), and potential niche competition has been suggested or seen in previous studies (Moyle and Senanayake 1984; Schut et al. 1984; Ajith Kumara and Amarasinghe 2008). However, in Western Ghats, India, Arunachalam (2000) observed that almost all streams were concurrently inhabited by *R. daniconius* and *D. aequipinnatus* and the similarity in abundance was within 25% of each other in eight out of ten streams. Therefore, the spatial interaction between these two species appears highly dependent on the spatial scale of studies with inhibition at small to intermediate scales *versus* co-occurrence at larger scales.

2.5 Developing suggested sampling times on reproductive development

2.5.1 Garra ceylonensis (Bleeker 1863)

The gonadosomatic index (GSI) of mature *G. ceylonensis* remained less than 2% of body weight across five sampling sites in the Uma-oya for both sexes in July and September (2009). Except for males of species that guard their eggs, a GSI of 1% body weight effectively discriminates mature individuals from immature fish in many species, and such a level is used as a criterion for eliminating immature fish from statistical analysis in the Canadian EEM program (Environment Canada 2010). *Garra ceylonensis* specimens in Ranamure-oya were smaller than the estimated maturity length of ~9 cm by De Silva (1991) and had a GSI of 0.82% (± 0.24 SD; n = 10). The size at maturity was estimated to be 8.5 cm of total length for females, and 7.0 cm for males, based on 95% of individuals belong equal to or above 1% GSI.

In this study, the average gonad size (GSI) for both sexes of *G. ceylonensis*– (18.1% (± SD 2.3) for females and 11.8% (± SD 1.3) for males) in the Uma-oya was higher in January, than July and September indicating that spawning in the Uma-oya occurs after January (Figure 2-2A).The large gonad size in females in January (mean GSI 18.1%, (range 6.1 to 27.9%) and the strong relationship of gonad weight to body weight (e.g. $r^2 = \sim 0.7$ ($p < 0.001$) in *G. ceylonensis* female in Erabadda) are consistent with synchronous single spawning species

(Barrett and Munkittrick 2010). Histological sections of *G. ceylonensis* female gonads during July and September revealed that the oocytes are at the primary stage of development, possessing Balbiani bodies with large nucleus under low power (10x10) light microscope (Tyler and Sumpter 1996). There were a few individuals with full gonad maturity (1 to 3 gravid females, and male with milky-white testes masses per site) (i.e. GSI >2%) in some of the sampling locations in July and September, 2009. The individual GSI estimates varied from 6.0 to 23.1% for these females, while they were from 6.2 to 11.8% for males, indicating that some individuals (<5%) still had mature gonads at later times of the year (Table 2-2).

There were clear differences in gonadal recruitment of *G. ceylonensis* in the Knuckles streams, and the Uma-oya stream. Gonadosomatic indices in *G. ceylonensis* found in the Knuckles streams (potential reference locations) in January, 2010 were much lower than in the Uma-oya. Within the mature individuals (total length >8.5 cm), GSIs above 2% were recorded in only about 14% (n = 29) in Theligam-oya, about 67% (n = 9) in upper Theligam-oya, and all individuals (n = 8) in Kalu-ganga were below 2% (Figure 2-3B). The minimum and maximum GSI values recorded from Theligam-oya (Rambukpitiya) and upper Theligam-oya (Etanwala) streams were 5.9 to 13.1% and 2.0 to 13.5%, respectively.

2.5.2 Devario malabaricus (Jerdon 1849)

Devario malabaricus female GSI values were consistently high throughout the year (Figure 2-4A) and, it is apparent that they can be sexually active throughout the year in the Uma-oya. The variability in GSI (coefficient of variation CV (as % of mean) and from the regression on gonad weight and body weight (r^2) of female and male *D. malabaricus* show erratic patterns (Table 2-3). Gonad development of male *D. malabaricus* was also similar through the year, with mean GSIs between 1 and 2 consistently at all sites during all seasons. The gonad development of female *D. malabaricus* was shown to be consistent over July through January, and GSIs were not statistically different during three sampling efforts ($p > 0.05$), although gonads appeared

larger in January in Girambe and Medawela (Table 2-3). Despite comparable GSI trends in females, macroscopic observation of female gonad masses in July specimens showed sparsely packed oocytes with more interstitial spaces than that of September. Further microscopic analyses are required to differentiate specific developmental phases occurring in gonads of *D. malabaricus* over shorter time scale.

Based on all mature female *D. malabaricus* collected from four Knuckles streams, the overall mean GSI of 2.7% (±2.0 SD), and the mean GSI value in all streams (min: 2.0%; max: 3.6%), was lower than compared to the Uma-oya stream. In January, >50% of mature female *D. malabaricus* were <2% GSI, while it was as high as 3.8% in the Uma-oya stream (Figure 2-4A and Figure 2-4B). This was dramatic, considering the variation of gonad recruitment in *D. malabaricus* between the Knuckles and the Uma-oya streams.

2.5.3 Rasbora daniconius (Hamilton-Buchanan 1822)

In this study, *R. daniconius* female gonads were relatively well developed in July (range of GSIs 4 to 17%), but gonad sizes were significantly larger in September (17.6%; range 9 to 21%) ($p = 0.003$), and remained high in January at Erabadda (range 7 to 20%), and smaller in February (range 7 to 11%) (Table 2-4). Other sites in the Uma-oya showed smaller sizes in January (mean 4.7%, range 1 to 12%). The mean GSI for male *R. daniconius* was 3.8% (in September), and did not significantly differ (Tukey test, $p > 0.05$) between months, although maximum gonad size was recorded in July (4.5%); maximum size in males often correlates with an early developmental size (Sundarabarathy et al. 2005). The regressions (r^2) of gonad weight and body weight were highly significant in all months ($p < 0.01$). Probably, male *R. daniconius* may be sexually active throughout the year as shown with other fish species, including the tinfoil barb, *Puntius schwanenfeldii* (McAdam et al. 1999).

Although *R. daniconius* is reported to be a short-lived species during which only a single spawning event occurs (Enderlein and Wickström 1991), the variability in female gonadal

condition was consistent between months (Figure 2-5A). The size of maturity in Erabadda (in the Uma-oya) was around 10 cm in length. The regressions of gonad weight and body weight were more highly significant in September ($r^2 > 0.85$), than July for both sexes (Table 2-4), and female *R. daniconius* showed less synchrony (high r^2 value) with male (low r^2 value) in July (2009) and January (2010), despite elevated mean GSI values. This high variability was also seen in January at two additional locations (viz. Diganathenna (DT) and Kottegoda (KG)). Despite a relatively high correlation of gonad weight and body weight ($r^2 > 0.60$), the GSI of female *R. daniconius* was significantly reduced ($p < 0.05$) in these two sites in relative to a comparable population in Erabadda (ER) in January (2010). Mean GSIs of male were also correspondingly low in both locations. In the overall scenario, there does not appear to be a single synchronized spawning period for *R. daniconius* in these areas.

Rasbora daniconius may be cued differentially for spawning in different locations. It was evident by contrasting individuals with GSIs lower (<3%) and higher (15.6% ± 3.8 SD) within the Uma-oya stream. Apparently, the spawning asynchrony may be extended across catchments, as seen in January (Figure 2-5A and Figure 2-5B). The egg masses (orange/ pale orange colour) were easily separated into individual eggs in all months in Erabadda. In contrast, the whole or fraction of female individuals were below 7% GSI in Diganathenna (100%; n = 8), Kottegoda (100%; n = 15), and Mathatilla (50%; n = 4), and the egg masses (yellow/ pale yellow colour) were not distinctly separated, in spite of a granular texture.

2.6 General biologies of selected fish species

2.6.1 Garra ceylonensis

The Ceylon Stone Sucker (*G. ceylonensis* Bleeker 1863) is a small, freshwater benthic fish species, that inhabits lotic environments with fast flowing water, and has a maximum age of 4 to 5 years (De Silva 1991). Previous studies indicate that *G. ceylonensis* is a single spawner

(De Silva 1991; Sundarabarathy et al. 2005), and it is reported to mature at a size of 7 to 9 cm, at an age of <2 yrs (De Silva 1991; Sundarabarathy et al. 2005), similar to the results found here. Spawning had been reported in July (Jones 1938), and December to March in Sri Lanka, with adhesive eggs being deposited on stony substrates (De Silva 1991). De Silva (1991) observed slightly above 8% of GSI in female *G. ceylonensis* at full maturity, during January and February in a stream population in the Sarasavi-oya (an upper Mahaweli tributary drainage). However, maximum gonad size is slightly higher in this study than that of De Silva (1991), and similar sizes were reported for *G. rufa* at full maturity (Esmaeili et al. 2005).

Garra spp. have not been widely studied in environmental monitoring assessments, although Marbaniang et al. (2010) studied chemical burdens in *G. lamta* near uranium deposits, and Dejenie et al. (2009) have shown a positive association between *Garra* biomass and total phosphorus in reservoirs. Adults feed on algal mats and periphyton attached to rock surfaces (Ojha and Singh 1992), and they attach to rock surfaces to feed by ventrally oriented adhesive organs and mouth feeding apparatus. Although De Silva (1991) thought that they were not migratory, Sri Lankan villagers in the Knuckles use highly crafted fish traps (*"kemana"*) made of twigs as fixed contrivances in tributary mouths to catch ascending fish of this particular species with the onset of heavy rains, as spawning times approach during November to December (Jayakody A. Sumith, pers. observ.). Moreover, *Garra* specimens tend to show jumping, creeping, and aggressive behaviour with sexual maturity (Sundarabarathy et al. 2005), which Silva and Davies (1986) linked with migratory movement upstream in order to spawn.

While the majority of sexually mature individuals in the Uma-oya stream were completing or near completion of their sexual investment in January, the main portion of the *G. ceylonensis* populations in the Knuckles streams were at the regression stage, implying possible heterogeneity in *G. ceylonensis* assemblages among sampling locations. Pethiyagoda (1991) reported that despite broader geographic distribution of *G. ceylonensis* (Jones 1938), *G. phillipsi*

Deraniyagala 1933 (Phillip's Garra) can be sympatrically occurring in hilly drainages of the Knuckles region (after Jones 1938). *Garra phillipsi* has shown almost no variation in taxonomical significant characters, and was thought to be a subspecies of *G. ceylonensis* (Pethiyagoda 1991). Further study refinements would be necessary in order to characterize and select target fish populations of *G. ceylonensis* to determine whether the differences in timing between the sites are due to habitat, genetic, or phylogenetic differences.

In Sri Lanka, *Maha* rainy season occurs from October to January, and *Yala* from April to July. De Silva et al. (1985) have shown that several small cyprinids inhabiting in southern highland streams in Sri Lanka are triggered for spawning with the onset of rainy season. Therefore, peak rainy months of May and November by default, are the major spawning months for several cyprinid (Chandrasoma et al. 1994; Sundarabarathy et al. 2004), and cichlid (De Silva and Chandrasoma 1980; Ward and Samarakoon 1981) species. It is apparent that *G. ceylonensis* is adapted to exploit the drier season during March to April after the northeast monsoon. De Silva (1991) hypothesized that *G. ceylonensis* is a dry season spawner, taking an adaptive strategy for more food (i.e. algae and diatoms) for larval survival. He reported that the breeding of *G. ceylonensis* was induced by water flow regime, rather than food availability. The post-spawning GSI (<2%) of *G. ceylonensis* in September in the Uma-oya suggests that gonadal investment must start during or after the month of October. In the two year study, De Silva (1991) observed annual upsurge to be occurring during the months of November and December, and peaked at January and February. In contrast, evidence suggests that *G. ceylonensis* in the Knuckles streams are primed for spawning by November to December. This was evidenced by the reproductively resting individuals in three streams in the Knuckles in January, 2010 (Figure 2-3B). Significance of differential environmental signaling for spawning of *G. ceylonensis* remains to be investigated.

2.6.2 Devario malabaricus

Life history information for *Devario malabaricus* is sparse (Froese and Pauly 2010). Although the sensitivity and testing value of *D. malabaricus* has not been explored, the closely related zebrafish *Danio rerio* has been used as a standard model organism for laboratory work on fish (Laale 1977; Mayden et al. 2007), and is a model test species in OECD chronic test guidelines (Hutchinson et al. 2006), and has been widely used for toxicity testing (Versonnen et al. 2004; Cook et al. 2005; Van der Ven et al. 2007; Xu et al. 2008).

Devario malabaricus' deep fusiform-body is as an adaptation to faster flowing waters (McClure et al. 2006), and feeding habits include in-stream aquatic insects (McClure et al. 2006) and crustaceans (e.g. shrimps) (Piet and Viverberg 1999). There are differences in the pigment pattern in juveniles and adults of *D. malabaricus* (McClure and McCune 2003), and the time to maturity is reported to be less than a year (Pethiyagoda 1991). *Devario malabaricus* appears to be a rain-induced spawner, spawning in batches of <200 eggs at a time (Pethiyagoda 1991); Jones (1938b) observed eggs and larvae during April through July in the Gowerawela-oya (an upper Mahaweli sub-tributary; 900 m above MSL). *Devario malabaricus* has additional physiological and morphological adaptations to torrent hill streams as with the adhesive nature of eggs, the speedy egg development, and the presence of the cement organ in young larvae (Jones 1938b).

The pattern of gonad development of *D. malabaricus* is seemed to be associated with multiple spawning fish species, and the present study confirmed the possibility of few spawning events per year, probably in association with (bimodal) monsoonal rainfall pattern. This offers one of the challenges in timing of sampling. The result of *D. malabaricus* is consistent with that of rainfall-induced spawning reported on most of the riverine cyprinids in Sri Lanka (Chandrasoma et al. 1994; Sundarabarathy et al. 2004). For *D. malabaricus*, spawning ahead of

a major rainfall season impending in October through December is strategically very important for brood dispersal and food security (Graaf et al. 1999; Coward and Bromage 2000).

Pethiyagoda (1991) reported that *D. malabaricus* is a widely distributed species in Sri Lanka, while its closest relative *D. aequipinnatus* (McClelland) is recorded in the Knuckles drainages (Pethiyagoda 1994). To date, there have been conflicting systems of identification with interchangeable notions between *D. malabaricus* and *D. aequipinnatus* McClelland (Pethiyagoda 1991; Pethiyagoda 1994), but detailed taxonomic studies on these "two forms" could not be found in the literature. In Sri Lanka, Moyle and Senanayake (1984) and Wikramanayake and Moyle (1989) specified *D. malabaricus* as deep-bodied species that inhabit fast-flowing hill streams. Alternatively, Sri Lankan freshwater fish literature used interchangeable names for giant danio as *D. aequipinnatus* (Schiemer and Hofer 1983; Silva 1993; De Silva and Somarathna 1994) and *D. malabaricus* (Jones 1938b; Weliange 2007). Graser et al. (1996) retained the *D. malabaricus* (Jerdon 1849) nomenclature over *D. aequipinnatus* implying that the latter might be a distinct species. However, during the current fish survey, some subtle morphological characters, such as varied pattern of vertical elements (McClure and McCune 2003), and partly analogous dorsal body curvature to another close relative *D. pathirana*, were observed in the Knuckles populations over the Uma-oya. Future studies would be necessary in order to understand the mosaic speciation, and species selection from a genetically homogenized population in impact assessments. It would be best to ensure that reference sites be selected within the same drainage where possible to avoid these issues.

2.6.3 Rasbora daniconius

Rasbora daniconius is a carnivorous species feeding on insects and zooplankton, and may be omnivorous (Schiemer and Hofer 1983; De Silva and Somarathna 1994; Piet and Vijverberg 1999). They are visual feeders (Pet and Piet 1993) with an up-turned, supra-terminal mouth, and a fusiform body shape, and forked tail (Ward-Campbell et al. 2005). Prem Kumar

and John (2007) reported that the most intensive feeding periods for *R. daniconius* are June and July, and October and November, which might be coinciding with active spawning periods (Litaay and De Silva 2003). This species has not been widely used in environmental monitoring studies, despite limited use in a few laboratory studies on acute and sub-lethal toxicity tests using heavy metals (Khangarot and Rajbanshi 1979), pesticides (Rastogi and Kulshrestha 1990; Wijeyaratne and Pathiratne 2006), and pulp and paper mill effluents (Pathan et al. 2009). Wijeyaratne and Pathiratne (2006) showed *R. caverii* to be markedly more sensitive to brain acetylcholinesterase depression compared to exotic tilapia *Oreochromis mossambicus*, suggesting that closely related species may be a good surrogate for agricultural effects monitoring.

Rasbora daniconius is reportedly a rain-induced spawner with opportunistic survival (Graaf et al. 1999; Lim et al. 1999). High resilience in terms of shorter population doubling period (<1 ½ yrs.) implies *R. daniconius* is a short-lived species (Froese and Pauly 2010). Fecundity has been reported between 929 and 7,398 eggs per female, and female ovary weight has been positively correlated (linearly) with both body weight, and curvilinearly with the standard length (Prem Kumar et al. 1985). Reproductively active individuals have been reported above 4.7 cm standard length (Prem Kumar et al. 1985). The species has a cyclic reproductive pattern with peak spawning occurring during July through August in India (Bhargava and Raizada 1973; Prem Kumar et al. 1985), and May through June in Cambodia, coinciding with the rainy season (Lim et al. 1999). It is reported to have a pre-spawning period from January to April, and post-spawning periods from September to December, and it can be assumed that Sri Lankan populations would be similar.

It has been reported that reproductive activity in teleost fishes is guided by necessary cues from the environment, which can be governed by the endogenous basis of physiology. Environmental cues may be acting as influential mechanisms to get synchronized their behaviour

with that of conspecifics (Helfman et al. 1997). Although several studies (listed above) report a relationship of spawning with rainfall in this species, Alkins-Koo (2000) listed other key proximal factors as spawning cues including changes in water chemistry, water temperature, flow rates, food supply, and availability of spawning sites. Ng and Munro (1997) have shown that reproductive cyclicity of several rasborines (viz. *R. elegans*, *R. heteromorpha*, *R. maculata*) was not influenced by water conductivity (or, in other words salinity), despite its possibility to play a role as synchronizing final maturation and spawning. The spatio-temporal reproductive variability of *R. daniconius* suggests a high amount of variability between sites, and that assessment programs using this species would have to select reference sites carefully.

It will be necessary to evaluate multiple species for their potential sensitivity; it is also important to examine potential sampling sites for the presence of rare, endangered, and threatened species that might affect sampling capabilities. In Sri Lankan rivers, there are a number of congeneric species (i.e. an organism belonging to the same taxonomic genus as another organism) that overlap in ranges, including *Garra phillipsi* with *G. ceylonensis*; *Devario pathirana* and *D. aequipinnatus* with *D. malabaricus*; and *Rasbora vaterifloris*, *R. wilpita*, *R. caverii* with *R. daniconius* (Wijeyaratne 1993). Many of these species are considered as highly threatened according to national assessments (MENR and IUCN 2007), since most are either endemic or confined to small scale localities. This listing includes two *Rasbora* spp. (*R. vaterifloris* and *R. wilpita*) that were not captured and *D. pathirana,* which was only captured in our preliminary studies in small numbers in the Kalu-ganga stream. The Sri Lankan Fisheries and Aquatic Resources Act No. 02 of 1996 have instituted export restrictions on three species, but there are no restrictions on the two *Garra* spp., or on *D. aequipinnatus*, *D. malabaricus*, and *R. daniconius* that were collected (Amarasinghe et al. 1996). The latest version of the IUCN Red List of Threatened Species (IUCN 2010; version 2010.1) declares that *D. malabaricus* and *R. daniconius* are of least concern from the conservation point of view, because they are very

common; however, the status of *G. ceylonensis* is imprecise, because of the wide distribution and potential presence of the congeneric species that has not been identified precisely. There are instances where there have been confusions in identification of congeneric species (Bambaradeniya et al. 2004; Weliange 2007). The basic life history strategies, such as timing and frequency of reproduction of the congenerics are poorly known or totally absent. The key information to help choose species for detecting impacts of stress on river systems are shown in Table 2-5.

2.7 Development of a strategy for defining sampling periods and sample size

There are three main factors to consider in selection of the optimum sampling periods including (a) an understanding of the temporal aspects of the stressors so that the periods of highest potential stress to fish can be optimally evaluated; (b) the seasonality of the reproductive development of the sentinel species; and (c) calculation of appropriate sample sizes for detecting meaningful differences.

Responses of fish to environmental stressors usually occur within a temporal or seasonal pattern. The hydrologic flow pattern is a major determinant of the stream chemistry across catchment scale in agricultural situations (Dayawansa 2003). Depositional sediments near urban and agricultural areas frequently contain organic and inorganic contaminants resulting in severely impacted water quality. In addition, pesticides can be transported off-site through spray drift and surface runoff (Wirth et al. 2001; Leight et al. 2005). Exposure to peak agriculture-related stressors in Sri Lankan rivers coincides with active growing seasons. In tropical climates, agricultural impacts are highly seasonal because seeding and/or planting coincides with rainfall patterns (Domroes 1979). In Sri Lanka, there are two rainfall patterns; the northeast monsoon (*Maha*) during the months of October through January, and the southwest monsoon (*Yala*) during April through July. The northeast monsoon is the main rainfall season covering the entire

island, and is the major cropping season, while the *Yala* is the minor rainfall season (Domroes and Ranatunga 1992). Following heavy rainfall events in the Uma-oya catchment, irrigable lowland fields are prepared for rice cultivation and sediment transport in streams during the period of land preparation is most often episodic, but persistent. The most appropriate fish sentinel species to monitor would be exposed in the highest levels of disturbance during the most sensitive developmental periods including during gonadal, egg and embryonic development.

The protection of the survival, growth, and reproduction of fish species is important as these aspects are indicators of aquatic health and the environment. Fish reproduction is the most challenging to evaluate and protect because it varies seasonally, and has the highest variability among the functional endpoints at the individual level. Furthermore, reproduction often involves migration and movements of fishes between habitats. The key aspects of reproduction to evaluate are the variability among individuals (since it will affect power and sample size requirements for study design), and the seasonal pattern (since it will affect the sensitivity and interpretability of results). A major challenge for developing countries in assessing fish health and developing sampling designs is the lack of background data on reproductive development of fish species. A sampling program can usually begin with an assessment during periods of active reproductive development. Critical information to know during this period is whether fish are synchronous in their development (Table 2-6), if they show variability across sizes, and whether there is any seasonality of the reproductive response (Rinchard and Kestemont 1996; Sundarabarathy et al. 2005). For tropical fishes, highly variable and episodic environmental fluctuations are unlikely and therefore multiple and protracted spawning events may be common (Schut et al. 1984; Chandrasoma et al. 1994; Tyler and Sumpter 1996; Sundarabarathy et al. 2004).

The bioassessment study design should calculate *a priori* the minimum sample size needed to detect whether a difference is present. This sample size can be calculated if decisions

are made about the level of α (level of significance required), β (power level required, where power = 1–β), the standard deviation (SD) of the endpoint of interest, and the critical effect size (CES; Munkittrick et al. 2009). The standard power analysis equation to determine sample size (n) (Green 1989) is:

$$n = 2(t_{\alpha} + t_{\beta})^2(SD/CES)^2$$

where:

t_{α} = value of Student's t statistic (two–tailed) with (n–1) df at a significance level of α

t_{β} = value of Student's t statistic (one–tailed) with (n–1) df at a significance level of β

Consideration of the power level requires balancing the bias of accepting an effect when there is no effect (Type I error), and concluding that there is no effect when there is in fact an effect (Type II error). The latter situation is undesirable from an environmental protection viewpoint. In this sense, optimizing the power required (1–β, where β is the probability of a Type II error) to an acceptable level would be necessary such as with $\alpha = \beta = 0.05$ resulting in 95% of power (Environment Canada 2010). A traditional 80% power ($\alpha = 0.05$; $\beta = 0.2$) is also accepted. In calculating the sample size requirements in the Canadian EEM program it is required to calculate three potential sample sizes, using ($\alpha = 0.05$, $\beta = 0.20$), ($\alpha = 0.10$, $\beta = 0.10$), and ($\alpha = 0.05$, $\beta = 0.05$) to evaluate the consequences of power decisions on required sample sizes. Traditionally, sample size calculations use the SD of female gonad size (GSI) since it is usually the most variable of the required endpoints, and a CES of 25% (Munkittrick et al. 2009). If sample size requirements are too high, then patterns of gonad variability are investigated to see if the SD can be reduced by focused sub-sampling of a specific component of the population or sampling during a different season, or re-designing the program using a different fish species. The key steps in selecting required sample sizes for study design are presented in Table 2-7.

2.7.1 Garra ceylonensis

Active gonadal development and spawning in *G. ceylonensis* coincides with peak rainfall events in November–December. Based on gonad development, January was the selected sampling month for *G. ceylonensis*. The optimal sampling period of January coincides with the *Maha* rainfall period making this species a strong candidate for evaluating the potential impacts of agricultural stress on river systems. The sample size requirements for those species were statistically evaluated in order to address the required critical effect size in gonad development from a minimum number of individuals (Table 2-8). Depending on power levels selected (80 to 95%), the sample size required to detect a gonad size difference in females was 10 to 30 adult individuals and 11 to 39 mature male individuals.

2.7.2 Devario malabaricus

The gonad development of female *D. malabaricus* was shown to be consistent across sampling seasons in July through January, and males were developed year round. The lowest variability was seen in September and January. Using the data of female *D. malabaricus*, at the minimum variance level of 23.8% in Puhulpola and the corresponding sample size requirement was 15 individuals ($\alpha = 0.05$; $\beta = 0.2$). For the purpose of the current assessment, the sample sizes required to detect a 25% difference in GSI were 15 to 29 for female, and 17 for males, depending on the location (Table 2-8).

The r^2 from the regressions of female gonad weight on body weight during the period of lowest variability (September) was less than half of that observed for *Garra* in their periods of lowest variability (January). This lower r^2 would be expected in a multiple spawning species, where there is variability between females in stages of egg development and proximity to the time of spawning (Sundarabarathy et al. 2005). There does not seem to be a priority sampling period for *D. malabaricus* in Sri Lanka, but the period of minimum variation of body weight related to gonad weight occurred in September for female *D. malabaricus* suggesting that they

were approaching a major spawning event (Table 2-3). Based upon the female and male *D. malabaricus* collected in September, the best sampling timing would be at the end of August or early September which is prior to the initiation of the *Maha* season and at the end of the dry season which ends typically in July.

2.7.3 Rasbora daniconius

Gonad weight and body weight were not always significantly related in *R. Daniconius*. For example in Kottegoda, female *R. daniconius* showed high correlation between gonad weight and body weight (ca. $r^2 > 0.9$; $p < 0.001$) despite the state of low gonad growth (ca. 2.7% GSI). The gonad weight – body weight relationship of *R. daniconius* was highly correlated (ca. $r^2 > 0.85$) for both sexes in September (Table 2-8) and the timing of sampling would be optimal in September for females, based on lower variability at this time.

Because of the low variability in gonad size (CV = 23.4%) for female *R. daniconius* in September, a 25% difference could be detected among individuals at $\alpha = 0.05$ and $\beta = 0.2$ using only 15 to 16 individuals. The low sample size requirement in September (female n = 6; male n = 9) in each sex means that this period would be a preferred sampling time for females, whereas males showed minimum variance in January and sampling would require 24 individuals per site.

2.8 Selection of reference sites

Ecological impact studies are often seen as comparing assessments for differences in means between reference sites and exposed sites. There are several design protocols that can be used to evaluate impacts of point source discharges in riverine environments (Kilgour et al. 2007), including upstream-downstream impact-control designs and before-after control-impact designs (BACI). These approaches require reference sites with similar variability for physical and chemical features that have the ability to readily differentiate the impact. There are alternate study designs available, such as stream gradient designs and reference-condition approach

(RCA) designs that also can be incorporated into the study design. The former design is more appropriate when suitable reference sites are not available and the latter is best where gradients and specific reference sites are unavailable (Reynoldson et al. 1997; Kilgour et al. 2007; Smiley et al. 2009). However, the RCA approach requires multiple reference sites (>20) and the variation among these sites can be greater than temporal variability (Bailey et al. 2007). For any selected study design to be successful, the selected variables must behave independently from the stressor of interest (agricultural impact in this study) for assessing an impact on biota (Bailey et al. 2007). A fish species that shows high variability in its life history characteristics would complicate the assessment. Historically, the RCA approach has been applied and tested with stream benthic invertebrates (Reynoldson et al. 1997; Reece and Richardson 2000; Bowman and Somers 2005), and less on fish (Kilgour et al. 2007). The approach when used in fish focuses on community as the response of interest rather than the population.

Selecting appropriate reference sites is the most difficult aspect of field study designs (Munkittrick 2009) and this selection becomes more complex with non-point stressors. Streams running across agricultural catchments are expected to be stressed along spatial and temporal gradients (Allan 2004). However, a situation where the stressors are diffusing (non-point source) such as observed in the Uma-oya catchment, increases the difficulty in finding reference locations within the catchment. Identifying reference sites in this case study is more complex because of the potential for alternate catchments to contain unidentified subspecies and the co-location of conspecifics. Ideally the best reference sites will be located within the study catchment itself and would incorporate a gradient design through areas of increasing exposure. An agricultural impact assessment study in Canada has explored stream gradients in forested upstream sites (reference), near field sites (transition zone), and impacted (downstream) locations (Gray 2003). Ibarra et al. (2005) considered altitudinal clusters in the Garonne basin in France for assessing impacts of non-point source pollutants on fish. However, such gradient

assessments have been justified with limited fish movements between experimental sites (Gray 2003). Therefore, if the distance between two sampling sites within a stream or tributary streams is large (i.e. located at considerable distances apart within a catchment), it may be acceptable for replicated assessments, as long as the abiotic factors are similar between these areas.

It is not always possible to design studies across large distances because of the presence of confounding factors (Munkittrick 2009). For example, human encroachment for agriculture has destroyed approximately one third of the forest (nature) reserve in the upper catchment of the Uma-oya (Environmental Foundation Limited 2007). In a major agricultural impact situation some streams may be subjected to one or more confounding influences. For example, one of the main tributary streams, Bomburuella-oya of the Uma-oya, has been influenced by both urban and domestic waste sources from the upstream town of Nuwara Eliya (6°58′0″N, 80°46′0″E) and also from a "sanitary" landfill (Jayakody A. Sumith, pers. observ.). The Uma-oya flows through a major town, Welimada (6°54′96″N, 80°55′22″E) which is another confounding source of environmental stressors for the downstream locations.

Smiley et al. (2009) advocated the use of replicated experimental designs with appropriate controls and treatments to represent the ideal study design. Conceptually, a stream gradient study design with reference locations upstream of the sources of contamination would be an ideal study design. Perhaps a better study design would compare magnitude of effects in streams that drain agricultural land to the effects in streams that drain forested landscapes. We identified that it was not ideal to design upstream reference sites in the Uma-oya catchment because the furthest upstream reaches of the system were void of fish, and there was considerable agricultural development in the other upstream reaches (Jayakody A. Sumith, pers. observ.).

2.9 Final study design

This chapter provides a conceptual study design for agricultural impact assessment applied to tropical countries using small-sized abundant fish species. In an appropriate study design, iterative sampling for re-examination and for confirmation of observations would be required in all steps of an effective bioassessment program (Munkittrick 2009). We propose a study design approach (Figure 2-6) starting with a common premise of ascertaining potential sentinel fish species (minimum of two species). Depending on the availability of reference sites within a study area, one of two design approaches could be considered. The longitudinal gradient designs classify reference sites of pristine or near pristine (un-impacted condition) along an upstream to downstream gradient, whereas cluster gradient designs classify sites from least-impacted upstream locations. For cluster gradient designs, stratification of most upstream sites in tributaries based on elevation (and/or stream size) would help to select the most appropriate group of reference sites (cluster) to which impacted sites are compared. General guidelines for selecting reference sites in river systems are also available in Hughes et al. (1985) and Dallas and Fowler (2000).

A variety of responses in fish populations can be detected using three functional attributes of "fish state" that can be impacted by stressors including survival, energy storage (change in food availability), and energy use (food conversion efficiency) (Gibbons and Munkittrick 1994). Such assessment tools are instrumental for teasing out "effects" of fish populations under stress.

An "effect" is a statistically significant response in an endpoint in a comparison of response measures in fish from reference areas (Munkittrick et al. 2000). For the "effect" to be clearly distinguished from natural perturbations (Munkittrick et al. 2000), the variability between reference and impacted sites should be greater than within site variability (Dubé 2003). However, extreme variability (due to low sample size, outliers, etc.) within cluster sites (e.g.

reference or impacted) is a malevolence often faced in biological experiments (Bosker et al. 2009) which needs to be addressed with proper experimental and statistical designs. Since, we are interested in the accumulated state of the stress or the stress experienced by fish inhabiting a particular locality, application of analysis of variance (ANOVA) and analysis of covariance (ANCOVA) for replicated sampling sites would also be feasible. Additional sampling sites should be considered if significant confounding variables are suspected. Provided the sites are randomly allocated, three sites for each confounding variable should be adequate for statistical justifications. When the "effect" is identified with proper statistical certainty, the magnitude and the direction of the effect relative to the reference condition, is taken for decision making in broader ecological, social, and economic terms (Munkittrick et al. 2000; Wirth et al. 2001).

2.10 Conclusion

There are recurring concerns relative to direct and indirect impacts of stressors on aquatic ecosystems by agricultural activities and associated chemical inputs (e.g. pesticides, fertilizer, etc.). The potential deleterious effects of stressors can be extended across various taxonomic groups including fish living in exposed environments. There are other concurrent threats to fish populations including sediment deposition and hydrologic changes, etc. The advent of intensive and year-round agricultural practices over several decades could have placed continued and persistent stress on fish populations compared to pristine environments. From the perspective of Harding et al. (1998), the present day fish community structure is a modified version of the past. Therefore, fish health assessments are challenging in any given locality as suitable environmental sentinels have to be selected from these systems that have been historically stressed. The challenge to proper study designs is further complicated as the existing fish assemblages are continually stressed over a long period of time, meaning that ecologically relevant effects may be very subtle (Ibarra et al. 2005), and genetic changes may have occurred

over time. Impact assessments are more consistent if comparisons are made between systems that are co-evolved (Moyle et al. 1998). The mosaic distribution of genetically different fish populations within a catchment may result in important consequences in the assessment designs and interpretation of data (Cowx et al. 2009).

My agricultural impact assessment is expected to be site-specific, and refinements would undoubtedly be necessary in the course of the investigation as more knowledge of the system and the species accumulates. The pre-sampling of the natural stream gradient in the Uma-oya revealed that suitable sentinels in terms of abundance were available, and that the composition of the fish assemblages was adequate. The current assessment incorporates a suite of fish species associated with water column and benthic habitats and varied levels of food habits. Fish distributions in potential reference locations (i.e. upstream) are either seriously depauperate (due to harsh environmental conditions), or mixed with sympatric species who's taxonomic and life history characteristics are largely undescribed. The magnitude and the timing of reproduction are not consistent between impacted and potential reference sites and that creates uncertainty in interpreting responses. Structured sampling with the current data support will expect consistent (low variance) data for stressor-based agricultural impact assessment in Sri Lankan streams. In the immediate juncture, some of the revelations encountered could be taken as the *status quo* and, when more data are available, risk assessment methods will be refined, and uncertainties will be reduced.

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Table 2-1. Summary of fish species presents in the Uma-oya (stem stream and tributaries) and the Knuckles streams.

Family/Species	Number of sites present (out of 12)[(a)]	Abundance (%)[(b)]	Max age (yr)	Food habits*	Spawning Habits**	Fecundity[(c)]	Country Status[ϕ0(d)]	IUCN Status[ϕϕ0(e)]	Resilience/ vulnerability[φ 0]
Cyprinidae									
Garra ceylonensis	11(4)	21.7-75.6 (5.9-84.5)	4-5	Detritus, Algae	Single (Total)	85-738[i] 740-4390[ii] 407-5902[iii]	ED	-	High/ Low-Moderate
G. phillipsi[ϯ]	?	?	?	Algae	?	?	ED	-	High
Devario malabaricus	9(4)	26.9-70.1 (15.5-61.0)	?	Insects, Detritus, Ephemeroptera larvae	Multiple (Protracted)	>200	IN	LC	High/Low
D. aequipinnatus[ϯ]	?	?	?	Insects, Annelids Crustaceans	Multiple (Protracted)	>1000	IN	-	Medium
D. pathirana	1(1)	(3.6)	?	Insects	?	?	[ED]/CR	EN	High
Rasbora daniconius	7(2)	1.7-45.6 (16.6-21.0)	?	Macrophytes, Zooplankton, Insects, Fish, Detritus	Single (Total) (Semelparous[i])	929-7398[iv] 3020-761[v]	IN	LC	High/Low
Puntius bimaculatus	10(3)	0.8-36.8 (1.1-17.2)	?	Algae, Detritus, Diatoms	Multiple (bi-modal)	82-2366[vi]	IN	-	High/Low
P. dorsalis	1(0)	0.1	?	Zoobenthos Macrophytes Fish eggs	Multiple (Protracted)	100-767[vii]	IN	-	

P. sarana	1(0)	0.1	?	Insects, Fish, Algae	?	?	IN	-	Medium/ Low-Moderate
P. kamalika	0(1)	(2.4)	?	?	?	?	ED	-	
P. martenstyni	0(2)	(10.1-13.5)	?	?	?	?	[ED]/CR	EN	
P. sinhala	0(1)	(3.0)	?	Benthos, Insects, Algae	?	?	IN	-	
P. srilankensis	0(1)	(0.3-1.2)	?	?	?	?	[ED]/EN	-	High/High
Tor khudree	4(2)	2.4 (0.5-10.7)		?	?	?	IN	EN	Low/High
Channidae									
Channa gachua	4(0)	0.1-7.2	>3	Insects, Larvae, Fish	Multiple (bi-modal) (Total)	389-2130[viii]	IN	-	Medium/ Low-Moderate
C. orientalis	0(1)	(2.6)	?	Insects, Crustaceans Fish	Mouth brooder	?	[ED]	-	High
Cobitidae									
Lepidocephalichthys thermalis	5(0)	0.2-27.6	?	Green algae, Detritus, Leaf debris, Chironomid larvae	?	?	IN	-	High/Low
L. jonklaasi	0(1)	(0.8)	?	?	?	?	[ED]/EN	EN	
Neomacheilus notostigma	2(1)	0.8 (5.2)	?	Plankton Detritus	?	?	ED	-	High/Low
Clariidae									
Clarias brachysoma	0(1)	(0.4)	?	Zoobenthos, Fish, Crustaceans, Tadpoles	Guarder Nester (male)	?	[ED]	-	Medium/ Low-Moderate

Osphronemidae									
Belontia signata	0(1)	(1.2)	?	Insects, Detritus	Guarder Nester (male)	500	[ED]	LR	High
Anguillidae									
Anguilla spp.	1(0)	0.3	10-20	Fish, Crustaceans, Molluscs	?	?	IN	-	Very low
Poeciliidae									
Poecillia reticulata	5(0)	0.9-7.7	?	Insects Zoobenthos	Multiple (Protracted) Viviparous	26[ix]	EX	-	High
Xiphophorus hellerii	2(0)	1.0-1.9	?	Crustaceans, Insects, Worms	Multiple Live bearer	20-200	EX	-	High
Cichlidae									
Oreochromis niloticus	1(0)	14.4	9-12.4	Macrophytes Phytoplankton Algae, Insects, Diatoms	Multiple (Protracted) Mouth brooder	289-1459[ix] 243-847[x]	EX	NT	Medium

[†]Evidence has been presented for occurrence of the two species in the Knuckles streams (Pethiyagoda 1991; Pethiyagoda 1994). In the present study, they were not categorically identified.

[(a)]Within brackets are the numbers of Knuckles streams (out of 4).

[(b)]Within brackets are the abundance/range of abundance in the Knuckles streams.

[(c)]Number of eggs; (i) Size 7.5–8.5 cm (Sundarabarathy et al. 2005); (ii) Size 10.5-14.1 cm (De Silva 1991); (iii) Size 11.6-16.5 cm (**This study**); (iv) Size 4.7-8.8 cm (Prem Kumar et al. 1985); (v) Size 9.6-12.1 cm (**This study**); (vi) Mean size at maturity 3 cm (De Silva et al. 1985); (vii) Mean size at maturity 5.3 cm (De Silva et al. 1985); (viii) Size 10.3-14.9 cm (De Silva 1991); (ix) Size 10-23 cm (Shalloof and Salama 2008); (x) Size 14-22 cm (Peña-Mendoza et al. 2005).

[(d)]IUCN (Sri Lanka) status: CR= Critically Endangered, EN= Endangered, ED= Endemic, IN= Indigenous, EX= Exotic, [ED] = Endemic fish species protected from prohibition or severe restriction from export under the Fisheries and Aquatic Resources Act No. 2 of 1998.

[(e)]IUCN-World status: LC= Least Concern, EN= Endangered, LR= Lower Risk, NT= Nearly Threatened.

[*] Schiemer and Hofer (1983); Moyle and Senanayake (1984); Wikramanayake and Moyle (1989); De Silva (1991); Pethiyagoda (1991); Piet and Guruge (1997); Marbaniang et al. (2010).

[**] De Silva and Kortmulder (1977); De Silva (1991); Enderlein and Wickström (1991); Pethiyagoda (1991); Litaay and De Silva (2003).

[ϕ] MENR and IUCN (2007) ; [ϕϕ] IUCN (2010); [φ] Froese and Pauly (2010)

Table 2-2. Spatial and temporal pattern of reproductive variables (GSI) and their relationship for female and male *Garra ceylonensis* in selected locations in the Uma-oya stream, Sri Lanka.

Sex	Sampling month	Site	Mean GSI (%)[†]	SD	CV (%)	n	Regression equation[††]	r^2	p- value
F	Jul	Erabadda	1.1[c]	0.4	36.7	14	y = 1.90x – 3.12	0.60	0.001
		Medawela	1.0[c]	0.3	35.1	35	y = 1.66x – 2.92	0.43	<0.001
		Girambe	1.2[c]	0.5	41.8	21	y = 1.06x – 2.01	0.47	0.001
	Sep	Erabadda	1.3[c]	0.5	39.9	29	y = 0.68x – 1.48	0.19	0.018
		Medawela	1.6[c]	0.9	56.7	21	y = 1.48x – 2.45	0.36	0.004
		Girambe	1.1[c]	0.5	42.1	41	y = 0.90x – 1.83	0.11	0.036
	Jan	Erabadda	15.8[b]	4.2	26.4	54	y = 1.14x – 0.99	0.67	<0.001
		Medawela	20.3[a]	3.9	19.3	56	y = 1.03x – 0.73	0.61	<0.001
		Girambe	18.9[ab]	4.9	25.9	42	y = 1.16x – 0.97	0.54	<0.001
M	Jul	Erabadda	1.1[c]	0.4	36.1	30	y = 1.10x – 2.12	0.30	0.002
		Medawela	1.0[c]	0.2	22.1	14	-	0.03	0.569
		Girambe	0.9[c]	0.3	38.2	24	y = 0.93x – 1.98	0.34	0.003
	Sep	Erabadda	1.2[c]	0.2	17.5	31	y = 0.94x – 1.86	0.67	<0.001
		Medawela	1.1[c]	0.5	46.3	8	-	0.03	0.899
		Girambe	1.3[c]	0.8	59.4	12	-	0.02	0.656
	Jan	Girambe	11.5[ab]	3.5	30.3	6	y = 1.32x – 1.31	0.66	0.050
		Puhulpola	10.7[b]	2.1	19.8	29	y = 0.92x – 0.87	0.79	<0.001
		Mathatilla	13.1[a]	3.1	23.9	8	y = 1.26x – 1.17	0.87	0.001

[†]Gonadosomatic index = 100[gonad weight/body weight]; F = Female; M = Male

Within a column, for each sex, mean GSI among sampling months presented by different superscript letters are significantly different (ANOVA, Tukey test $p < 0.05$).

[††]Relationship of body weight to gonad weight (both variables were $\log_{10}$ transformed)

Table 2-3. Spatial and temporal pattern of reproductive variables (GSI) and their relationship for female and male *Devario malabaricus* in selected locations in the Uma-oya stream, Sri Lanka.

Sex	Sampling month	Site	Mean GSI (%)[†]	SD	CV (%)	n	Regression equation[††]	r^2	p-value
F	Jul	Girambe	4.4^{c}	1.8	39.7	24	y = 1.45x – 1.63	0.46	<0.001
		Medawela	5.8^{bc}	3.2	54.5	23	y = 1.96x – 1.85	0.72	<0.001
		Puhulpola	6.3^{ab}	2.0	31.7	41	y = 1.72x – 1.65	0.79	<0.001
	Sep	Girambe	6.4^{ab}	2.1	33.6	18	y = 1.16x – 1.30	0.30	0.018
		Medawela	7.1^{ab}	2.1	30.0	16	y = 1.21x – 1.30	0.23	0.058
		Puhulpola	6.4^{ab}	1.5	23.8	33	y = 1.03x – 1.23	0.49	<0.001
	Jan	Girambe	7.9^{ab}	3.7	47.3	16	-	0.20	0.086
		Medawela	8.2^{a}	3.3	39.8	19	y = 1.16x – 1.24	0.20	0.054
		Puhulpola	6.4^{ab}	2.4	36.7	17	y = 1.01x – 1.22	0.27	0.032
M	Jul	Girambe	1.1^{ab}	0.5	46.1	36	y = 0.95x – 1.97	0.30	0.001
		Medawela	1.7^{a}	1.0	59.2	45	y = 1.15x – 1.89	0.46	<0.001
		Puhulpola	1.3^{ab}	1.0	78.9	24	y = 1.10x – 2.01	0.24	0.016
	Sep	Girambe	1.1^{ab}	0.9	80.0	21	-	0.10	0.161
		Medawela	1.8^{a}	0.5	25.5	20	-	0.11	0.154
		Puhulpola	1.3^{ab}	0.6	45.1	29	y = 1.20x – 2.07	0.49	<0.001
	Jan	Girambe	1.6^{a}	0.6	36.8	18	y = 1.79x – 2.37	0.59	<0.001
		Medawela	1.2^{ab}	0.4	36.6	10	-	0.15	0.272
		Puhulpola	1.2^{ab}	2.1	43.0	9	-	0.24	0.184

[†]Relationship of body weight to gonad weight: Gonadosomatic index = 100[gonad weight/body weight]; F = Female; M = Male
Within a column, for each sex, mean GSI among sampling months presented by different superscript letters are significantly different (ANOVA, Tukey test $p < 0.05$).
[††]Relationship of body weight to gonad weight (both variables were $\log_{10}$ transformed)

Table 2-4. Spatial and temporal pattern of reproductive variables (GSI) and their relationship for female and male *Rasbora daniconius* in selected locations in the Uma-oya stream, Sri Lanka.

Sex	Sampling month	Site	Mean GSI (%)†	SD	CV (%)	n	Regression equation††	r^2	p-value
F	Jul	Erabadda	11.5^{b}	5.0	43.8	13	$y = 2.28x + 2.42$	0.51	0.006
	Sep	Erabadda	17.6^{a}	4.1	23.4	6	$y = 0.95x + 0.74$	0.91	0.013
	Jan	Erabadda	15.6^{ab}	3.8	24.2	11	$y = 1.56x - 1.47$	0.67	0.002
		Diganatenna	2.3^{c}	0.5	22.1	8	-	0.26	0.196
		Kottegoda	2.7^{c}	1.6	59.0	15	$y = 1.80x - 2.32$	0.91	<0.001
M	Jul	Erabadda	4.5^{a}	2.2	48.5	27	$y = 2.15x - 2.36$	0.31	0.002
	Sep	Erabadda	3.8^{ab}	2.8	76.0	9	$y = 3.30x - 3.13$	0.88	<0.001
	Jan	Erabadda	4.0^{ac}	1.2	30.4	15	$y = 2.54x - 2.83$	0.68	<0.001
		Diganatenna	2.8^{cd}	1.2	44.5	17	$y = 1.32x - 1.89$	0.52	0.001
		Kottegoda	3.0^{cd}	1.1	37.1	19	$y = 1.14x - 1.69$	0.64	<0.001

†Relationship of body weight to gonad weight: Gonadosomatic index = 100[gonad weight/body weight]; F = Female; M = Male
Within a column, for each sex, mean GSI among sampling months presented by different superscript letters are significantly different (ANOVA, Tukey test $p < 0.05$).
††Relationship of body weight to gonad weight (both variables were $\log_{10}$ transformed)

Table 2-5. Basic information to help choose species for detecting impacts of stress on river systems.

1. Background information and preliminary sampling.
a. What species are there?
b. How abundant are they?
c. Are the common species endangered or threatened, or are endangered or threatened species present that might affect sampling design?
d. Is there data available on longevity? Want species that live <10–15 years.
e. When are they there?
f. Are they likely to migrate?
g. Is the size distribution different in spawning seasons and non-spawning seasons?
h. Are all life stages present?
2. Feeding – prefer one that feeds on benthos (invertebrate feeders may move less than algal grazers?). Don't want a piscivorous species unless it is an ambush predator with a nest. Don't want a plankton feeder.
3. Size or age to maturation.
a. When do they spawn?
4. Impact on design will depend on issue. Do not want a species that spawns during high flows. Prefer one that develops gonads during periods of lower flow – effluent is in higher concentration during critical periods.
a. Is it known where they spawn (main-stem, tributaries, on vegetation, guarding, etc.)?
b. Is there information on pollution tolerance available?
5. Initial data analyses to assist in study design and limit unnecessary lethal sampling.
a. Are there sex differences in size and condition?
b. Can condition be used to separate sexes or immature fish?
c. Does GSI correlate with condition? (Are fatter fish fatter because they have bigger gonads?) – If so, you may be able to use this to separate sexes or reproductive stages.
6. Reproduction
a. Are there differences between males and females in GSI?
b. At what size (and age) does maturity occur?
c. Do you know how many times a year reproduction happens? – Do female ovaries look like they are multiple spawners?
7. Liver size – Are there sex differences or seasonal differences?

Table 2-6. Phenotypical differences between synchronous and asynchronous spawners as reviewed by Tyler and Sumpter (1996).

Process	Synchronous spawners		Asynchronous spawners
Gonadal weight in relation to body weight	High (usually 18 to 25%)		Low (usually 2 to14%)
Gonadal weight in relation to body weight (r^2)$^{\phi}$	>0.85 (single spawn)		NS
	Multiple spawners (few spawns) $0.4<r^2<0.85$	Multiple spawners (many spawns) <0.4	
Oocyte development in the ovary	Maximum of two cohorts at any time		Heterogeneous population of more than 3 or more stages
Size of oocytes	Large		Small
Spawning	Annual event or single occurrence in life		No apparent cyclicity (annually or otherwise); couple of events in a year/season
Vitellogenin levels in blood during oocyte development	High (50 ml^{-1})		Low (<1 ml^{-1})
Vitellogenin levels after ovulation	Sequestered prior to spawning (especially in fishes spawn once and then die)		Persistent at lower levels until metabolized

NS = not stated

$^{\phi}$ Barrett and Munkittrick (2010)

Table 2-7. Steps in selecting required sample sizes for study design.

1. Identify period of maximal GSI.
a. Are all fish mature at the same time?
b. Usually <<1% GSI can be used (for many species, but not all) to differentiate between immature and non-spawning fish; non-spawning should be closer to 1%.
c. If mature male GSIs are <1% at spawning, it suggests that they may be nesting or guarding species.
2. What is the strength of the gonad weight: body weight relationship?
a. $r^2 < 0.9$ means that you need many more fish.
3. When is the period of the lowest coefficient of variation for GSI?
a. May be able to use $r^2 > 0.9$ during maximum reproductive development to select synchronous spawning species.
b. Is GSI consistent across fish lengths? (Do smaller fish have a smaller GSI than larger fish? - suggesting older fish may spawn more often than smaller fish).
4. Select *alpha*, *beta*, critical effect size.
a. Evaluate at 0.05, 0.10 for *alpha*, 0.05, 0.10, 0.20 for *beta*, CES of 25% for GSI.
b. Is required sample size reasonable?

Table 2-8. Numbers of fish required to detect 25% difference in gonad development at different levels of power for female and male *Garra ceylonensis*, *Devario malabaricus*, and *Rasbora daniconius* at specified months.

Species	Sex	Sampling time	Site	Number of fish at specified power (1–β) and significance level (α)†		
				0.8/0.05	0.9/0.10	0.95/0.05
Garra ceylonensis	F	January	Erabadda	19	20	30
			Medawela	10	11	17
			Girambe	16	17	25
	M	January	Girambe	24	26	39
			Puhulpola	11	12	17
			Mathatilla	15	17	25
Devario malabaricus	F	September	Girambe	29	32	48
			Medawela	24	26	39
			Puhulpola	15	16	25
	M	September	Medawela	17	19	28
		January	Medawela	35	38	57
			Girambe	35	38	57
Rasbora daniconius	F	September	Erabadda	15	16	24
		January	Erabadda	16	17	26
	M	January	Erabadda	24	26	39

†α– probability of committing Type I error; β– probability of committing Type II error.

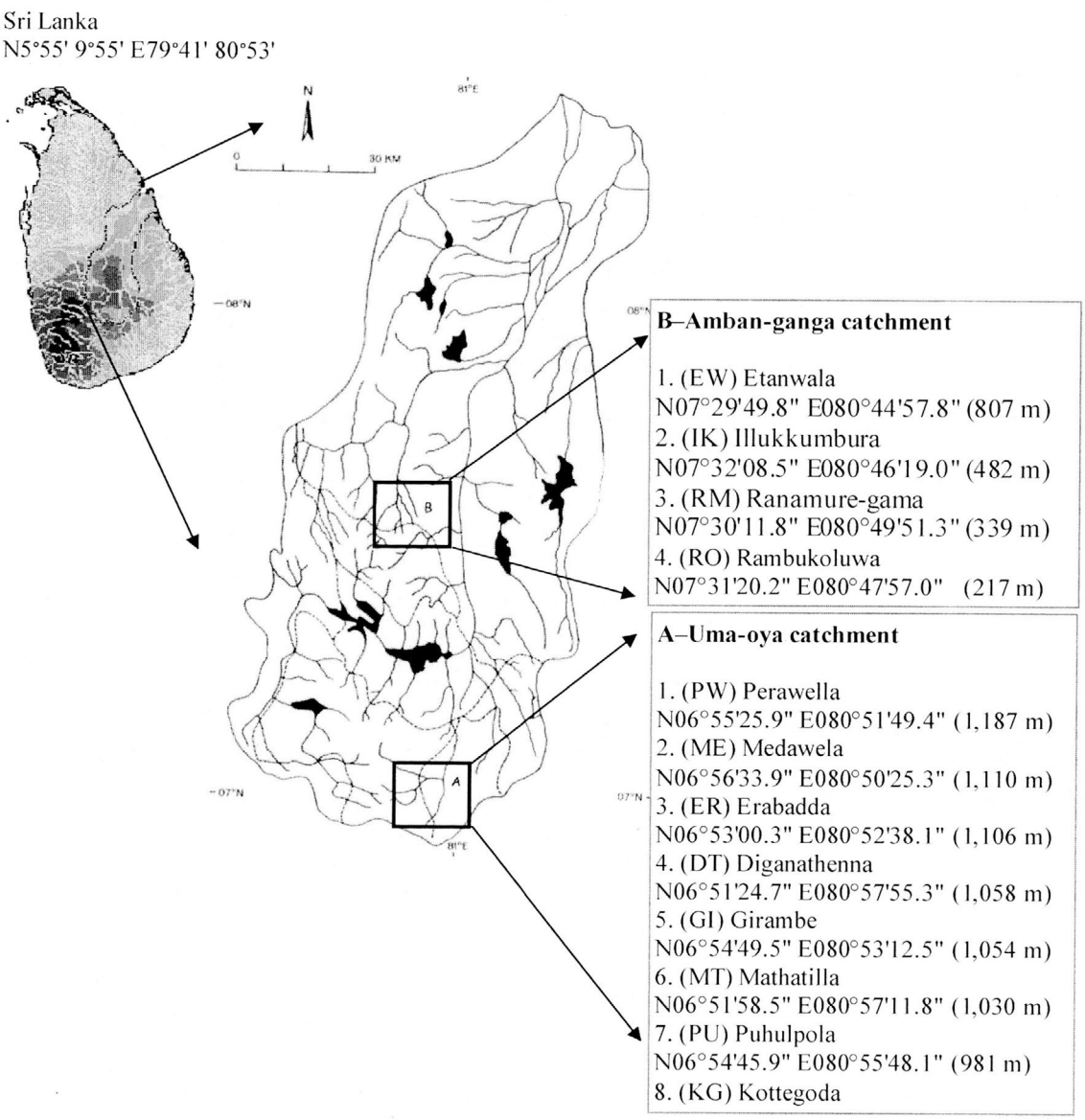


Figure 2-1. Map of major stream distribution and sampling locations in the Mahaweli river basin. (A) Uma-oya catchment, stream and sampling locations, (B) Amban-ganga catchment, (Knuckles streams) and sampling locations (Modified from Silva 1993).

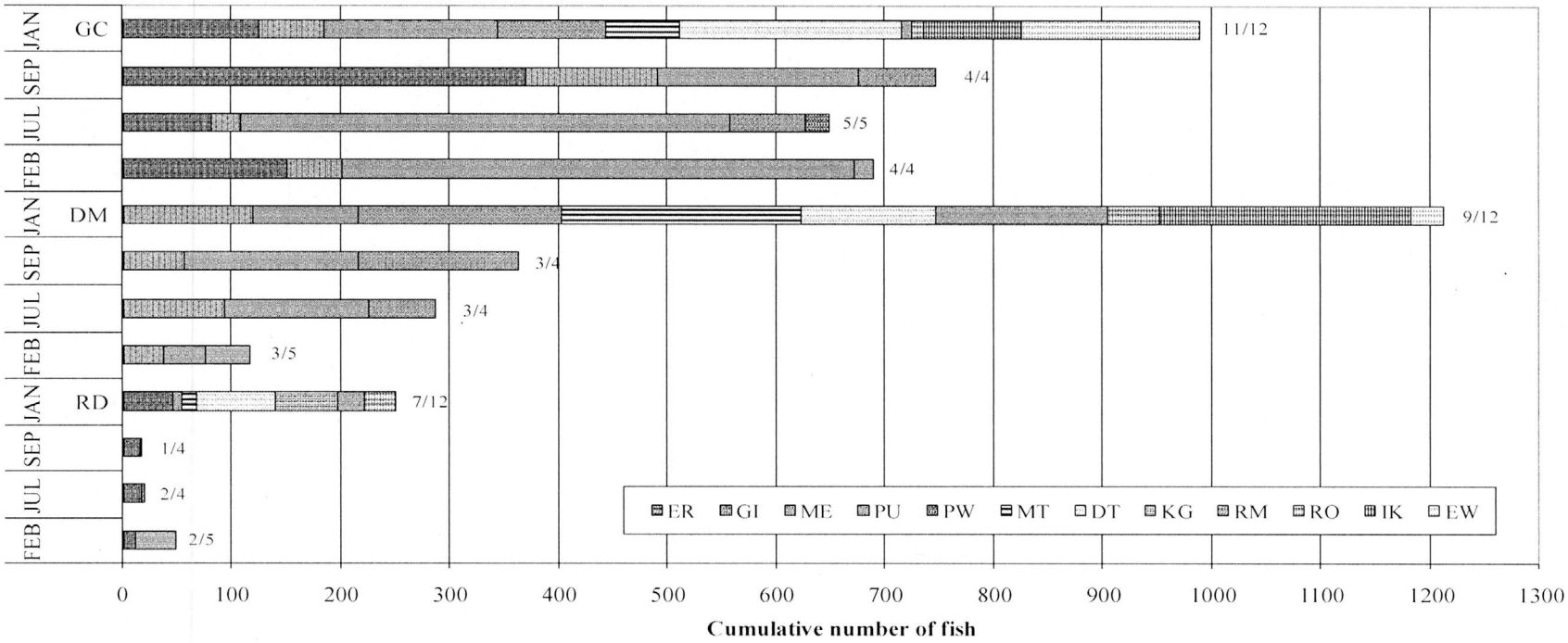


Figure 2-2. Seasonal abundance of *G. ceylonensis*, *D. malabaricus* and *R. daniconius* in the tributary streams of the Uma-oya and Amban-ganga catchments (Knuckles streams) of the Mahaweli River basin in Sri Lanka. Sampling in February (2009) and July (2009) by electrofishing technique and September (2009) and January (2010) by composite nets. January and February sampling months corroborated with "*Maha*" season (immediately after northeast monsoon rainfall); July and September sampling months corroborated with "*Yala*" season (immediately after southwest monsoon rainfall). For descriptive site codes, see Figure 2-1 legend; GC = *Garra ceylonensis* (Bleeker); DM = *Devario malabaricus* (Jerdon); RD = *Rasbora daniconius* (Hamilton-Buchanan). In situations where repeated sampling is involved, same stream reach lengths were maintained. The number ratio on the top of the bar represents the number of sites fish species present to the total number of sites sampled.

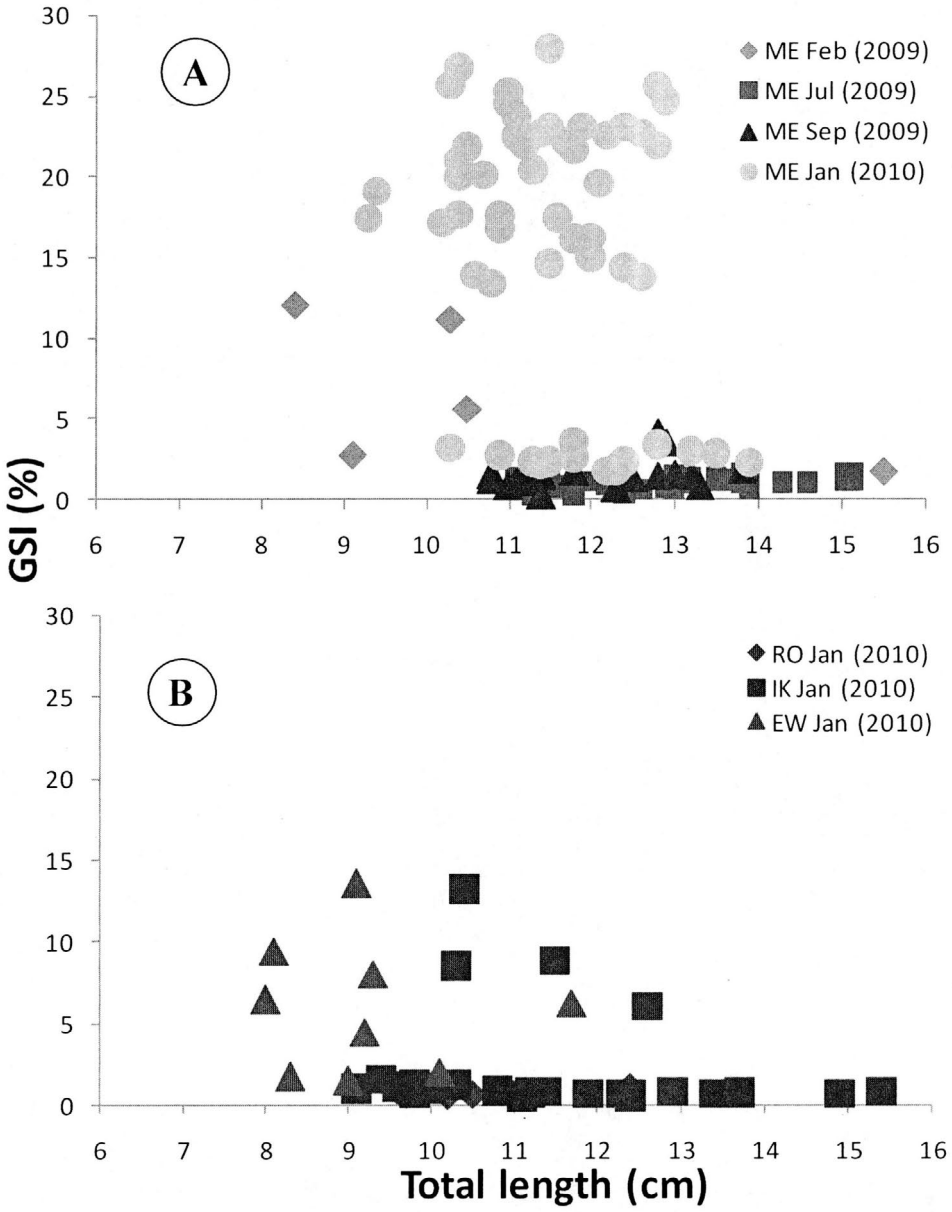


Figure 2-3. Scatter plot of gonadosomatic index [100* (gonad weight/body weight)] versus total length of female *Garra ceylonensis*. (A) Uma-oya locations (B) Knuckles streams in January, 2010. For descriptive site codes, see Figure 2-1 legend.

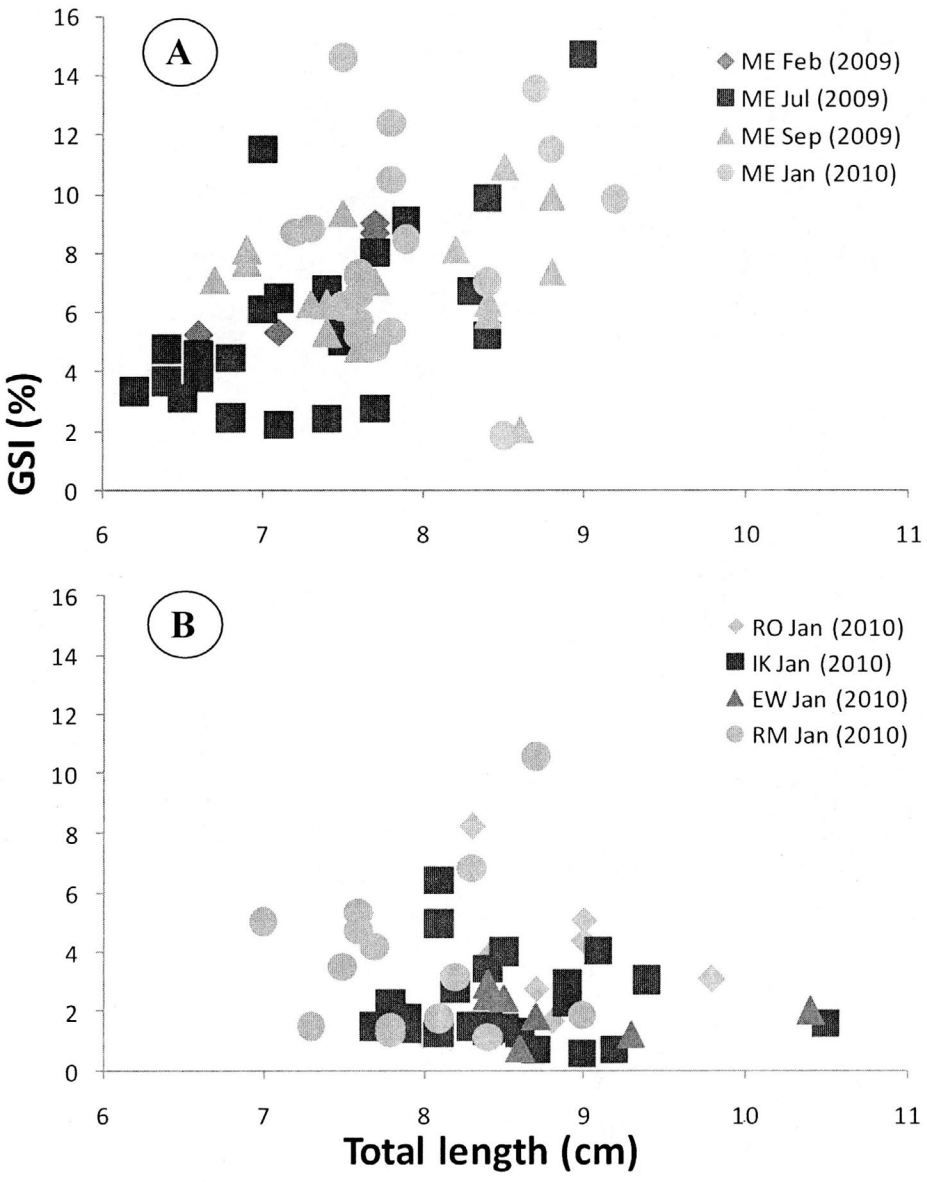


Figure 2-4. Scatter plot of gonadosomatic index [100* (gonad weight/body weight)] versus total length of female *Devario malabaricus*. (A) Uma-oya locations (B) Knuckles streams in January, 2010. For descriptive site codes, see Figure 2-1 legend.

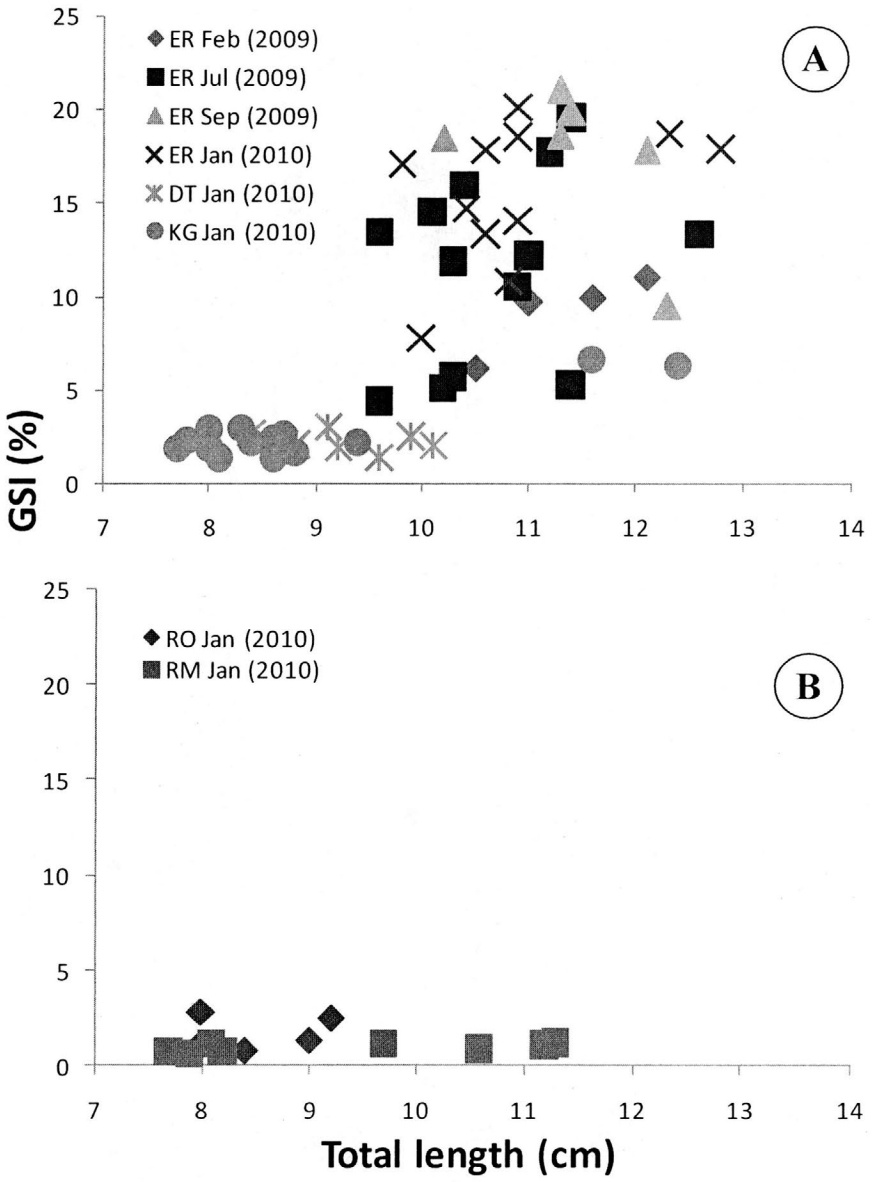
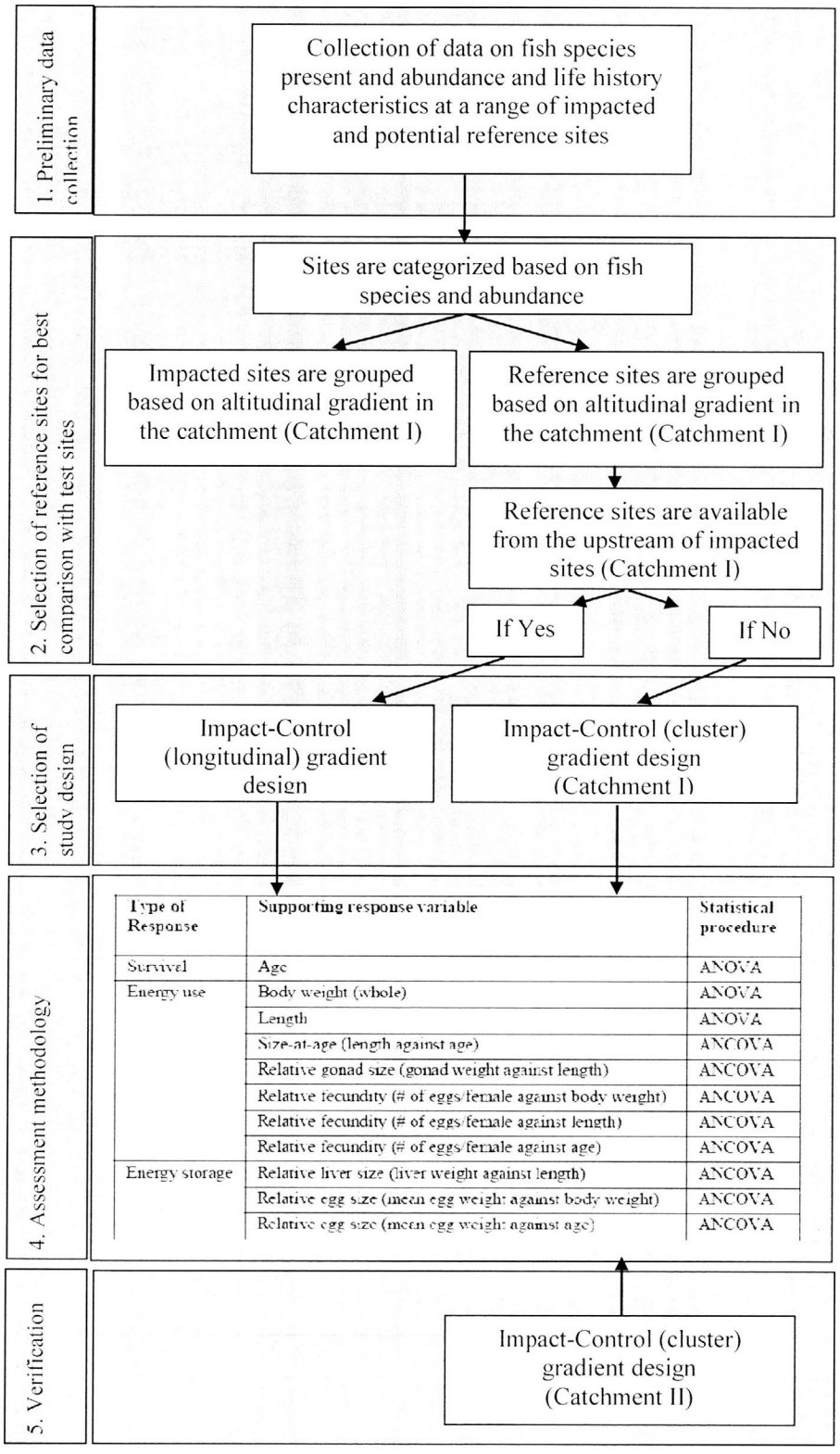


Figure 2-5. Scatter plot of gonadosomatic index [100* (gonad weight/body weight)] versus total length of female *Rasbora daniconius*. (A) Uma-oya locations (B) Knuckles streams in January, 2010. For descriptive site codes, see Figure 2-1 legend.



Type of Response	Supporting response variable	Statistical procedure
Survival	Age	ANOVA
Energy use	Body weight (whole)	ANOVA
	Length	ANOVA
	Size-at-age (length against age)	ANCOVA
	Relative gonad size (gonad weight against length)	ANCOVA
	Relative fecundity (# of eggs/female against body weight)	ANCOVA
	Relative fecundity (# of eggs/female against length)	ANCOVA
	Relative fecundity (# of eggs/female against age)	ANCOVA
Energy storage	Relative liver size (liver weight against length)	ANCOVA
	Relative egg size (mean egg weight against body weight)	ANCOVA
	Relative egg size (mean egg weight against age)	ANCOVA

Figure 2-6. Flowchart of assessment methods using impact-control design.

CHAPTER 3

3 Fish assemblage structure of two contrasting stream catchments of the Mahaweli River basin in Sri Lanka: hallmarks of human exploitation and implications for conservation [2]

3.1 Abstract

Patterns of fish community composition in the Mahaweli ichthyological region of Sri Lanka were examined in agricultural tributaries of the Uma-oya catchment of the upper Mahaweli River, in comparison to more pristine streams in a nature reserve in the Amban-ganga catchment. The Uma-oya catchment shows characteristics commonly observed in extensive agricultural exploitation, such as impaired water quality, and altered riparian vegetation. The most abundant fish species in the two regions were *Garra ceylonensis*, *Devario malabaricus*, and *Rasbora daniconius*, although their relative abundance differed between sites. *Garra ceylonensis* and *Neomacheilus notostigma* were the only endemic fish species in common, but the latter has been extremely depauperate. Endemism is higher in the reference sites (62.5%) than agricultural sites (ca. 25%); some of the reference streams showed greater diversity with unique fish species, and a few species that have not been recorded previously in the catchment. The ichthyofaunal similarity between two catchments was 39%, and fish species diversity was negatively correlated with stream gradients (Pearson's correlation -0.630; $p = 0.028$). Species density and biomass of *G. ceylonensis* and *D. malabaricus* were positively associated with phosphate and nitrate concentrations in study streams. The presence of exotic fish species were amongst the primary discriminants between human-exploited, and least-exploited (reference)

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streams. The possible negative impact of exotic *Oreochromis niloticus* (Nile tilapia) on indigenous *G. ceylonensis* is highlighted. This paper provides impetus for making several hypotheses for sustainability of stream fish assemblages in highly exploited agricultural catchments in Sri Lanka.

3.2 Introduction

Sri Lanka is an island (65,662 km^2) off the southern tip of the Indian peninsula between latitudes 5"55' and 9"55' N and longitudes 79"41' and 80"53' E. The central cross section of the island is characterized by a three-peneplane altitudinal model (Vitanage 1970), which divides prominent slope breaks into coastal lowlands (up to 270 m and 0 to 15 degrees slope), uplands (270 to 1,060 m altitude and 10 to 35 degrees slope), and highlands with well-defined high plains, and plateaus (910–2,424 m). This geomorphology gives rise to 103 radially draining perennial river systems, which can be grouped based on fish faunal distribution into six major river basins: Mahaweli, Kelani, Kalu, Gin, Nilwala (Pethiyagoda 1991), and Walawe (Jinadasa et al. 2003).

Sri Lankan streams and rivers house a diverse fish assemblage comprising 34 families from freshwater and saltwater ecosystems (Pethiyagoda 1991, 1994). The fish assemblage is not as diverse as other tropical countries (Fernando 1984), but it shares ancestral fish assemblages common to Asia. All of the 19 freshwater fish families recorded (Pethiyagoda 2006; Amarasinghe and Weerakoon 2009) also occur in the southern regional part of Western Ghats (Dahanukar et al. 2004) (i.e. the closest landmass of India). Differences in composition compared with the higher diversity in Western Ghats (25 families), may have appeared during the geological separation in Pleistocene ages (Wikramanayake 1990). This supports the scenario that these two areas remained in contact before their earlier separation, and remained isolated for a long time, in support of a distinct fish assemblage (i.e. endemic) in this continental Island.

Pethiyagoda (1991) described Sri Lanka's 109 freshwater and brackish species, and found cyprinids the most abundant (41, including 24 introduced fish species; Pethiyagoda 2006). Of the 85 native freshwater species, 38 are endemic to Sri Lanka (Bahir and Gabadage 2009), and 19 (49%) of them are cyprinids, while the rest belong to 25 other families. The taxonomic identification of freshwater fish species is not complete, and several indigenous cyprinids known to occur in Western Ghats (India) (Dahanukar et al. 2004), have been identified as new species in Sri Lanka (Meegaskumbura et al. 2008; Pethiyagoda et al. 2008; Silva et al. 2008). A most recent update of freshwater and brackish water fish species in Sri Lanka is presented in Appendix 1.

There have been few fish surveys along gradients in Sri Lankan streams, and the dominant fish distribution pattern in the highland plateaus, is unknown. Studies on freshwater fishes in Sri Lanka have been limited to commercial fisheries, and even these have been largely restricted to some of the man-made reservoirs, such as Parakrama Samudra (Duncan 1984), and Tissawewa (Piet and Guruge 1997). In a study of the longitudinal gradient in the upper Walawe River basin from an elevation of 200 m up to 2,000 m above mean sea level (MSL), the diversity and abundance of fishes diminished with ascending altitude, where maximum elevation for occurrence of indigenous fishes was 1,400 m above MSL (Jinadasa et al. 2003). A recent comparative study (Weliange 2007) on riverine fish assemblages in dry and wet zones in Sri Lanka revealed varied assemblage composition along the longitudinal gradient, with abundance and assemblage structure varying along the two streams, due to habitat characteristics. This implies that there are few potential environmental fish sentinels in streams at higher elevations; more importantly, the endemic species are limited in these areas, to less than six (Moyle and Senanayake 1984).

In small islands like Sri Lanka where freshwater fish species are limited, threats can be overwhelmingly detrimental to the overall conservation agenda. Of the 85 freshwater species

listed by Pethiyagoda (2006), 29 freshwater fish species are listed as threatened, and 10 as highly threatened, in the context of the national fish conservation agenda. Streams and rivers (i.e. lotic environments) hold two-thirds of the endemic species, and they account for more than 90% of threatened or vulnerable fish species (Wijeyaratne 1993). The indigenous fish base is threatened by multiple disturbances including water pollution, sedimentation, deforestation, water diversion and impoundments, exotic fish introductions, and over-exploitation (Wijeyaratne 1993; Silva 1993; Pethiyagoda 1994). Collares-Pereira and Cowx (2004) reviewed various threats to freshwater fishes, and ranked species introductions as the leading threat. Of the other potential threats, there is an increasing focus on pesticides in Sri Lanka, although the majority of studies have been conducted in the laboratory (Pathiratne and Ranasinghe 1992; Pathiratne and Athauda 1998; Pathiratne 1999), and few are based on real exposure environments (Wijeyaratne and Pathiratne 2006; Jayasundara and Pathiratne 2008; Pathiratne et al. 2009).

Fish population strength and dynamics in streams and rivers are essential components in study designs for fish conservation and management. In this study, we describe the community structure of fish species in streams in two river catchments in Sri Lanka: one with a history of intensive agricultural exploitation, as well as the least exploited catchment in the Mahaweli River basin in a nature reserve. We present results of seasonal sampling using two methods of fishing techniques conducted during February (2009) through January (2010). This preliminary study was designed to ascertain spatial and temporal distribution of abundant fish species in potential river catchments, for designing agricultural impact studies on riverine fish species, and for collecting baseline reference data.

3.3 Materials and Methods

3.3.1 Study area

The Mahaweli River is the longest river (335 km) with the highest discharge in Sri Lanka, and a catchment area of approximately 20% (10,327 km^2) of the total area of the country (Silva and Davies 1986; Silva 1993). The upper catchment covers approximately 25% of the central highland in Sri Lanka, with more than five distinct basins; the Uma-oya basin is the largest (Ranasinghe et al. 2008). The mean annual discharge is >14.5 (± 2.8 SD) million m^3 (as measured at the Welimada gauging station during 1993 through 2004; Mahaweli Authority, Sri Lanka). The catchment receives 1,570 to 2,180 mm of rainfall, annually (as measured in six stations within the catchment during 2008 and 2010; Department of Meteorology, Sri Lanka). The temperature maximum 28.7°C and minimum 16.2°C were recorded during 2008 and 2009 in the catchment. In addition to agriculture, the catchment is compounded with chanalization for irrigation, and stresses associated with a fishery, tourism, hydro-electricity, and water supply for domestic, public and urban necessities. Twelve sites were selected for the collection of baseline information. The sites were represented by eight locations (i.e. agriculturally impacted sites) of the Uma-oya catchment (stem stream), and its tributary streams, and four potential reference locations from the Amban-ganga upper catchment. The Amban-ganga headwater streams are located in the Knuckles region draining to the Mahaweli River; Kalu-ganga (river) and Kambarawa-ganga are separate drainage systems in the Mahaweli River basin (Figure 3-1,Table 3-1). The Knuckles is covered with montane forests and an associated buffer zone of approximately 260 km^2 area, which spans from 200 to 1,900 m above MSL (Pinard et al. 2010). The Knuckles streams represent a part of Amban-ganga catchment, which receives 1,435 to 2,111 mm of rainfall, annually (as measured in six stations within the catchment during 2008 and 2010). The maximum temperature of 31.5°C and minimum of 20.2°C was recorded during 2008 and 2009 (Department of Meteorology, Sri Lanka). Intensive human activities, such as

farming by slash-and-burn cultivation, fishing and hunting wild animals, and logging and deforestation in most of the areas have been diminished after the declaration of the area as Conservation Forest in May 2000, by the Government of Sri Lanka. A superficial estimation using a topographical map (1: 63,360; Survey Department, Sri Lanka, 1995) revealed that sampling locations in both catchments were falling into 3rd and 4th river order.

The upper reaches in both catchments are distinctly different from downstream locations, with cascading and down-pouring rapids with steep slopes. The stream sections of other sites in the Uma-oya were gently sloping tributaries with slow to moderate rapids, runs, and water stagnant pools. All sampling sections were composed of riffles, runs, cruises, and some pools made when water flow was blocked by boulders. The surrounding topographies of all sections in the Uma-oya were extensively utilized, and riparian areas were primarily cleared for vegetable cultivation. When riparian vegetation did exist, it consisted primarily of giant cane (*Arundo donax*), which grows up to 5 to 6 m height, interspersed in rare instances with bushes and taller trees. The giant cane is often used for fences and trellis, and during harvesting the stream is blocked by giant cane clearings, and floating remnants can often be seen. The surrounding vegetation in reference streams was a dense rain forest with a tall canopy, devoid of immediate human impact (Table 3-1).

3.3.2 Fish community assessment

Quantitative sampling was conducted along pre-determined sections from the Uma-oya (stream), and Amban-ganga catchments in February, July and September (2009) and January (2010). Sampling was always started from the lower (downstream) end of the stream section, and proceeded upstream encompassing every geomorphic channel unit, i.e. pools, runs, and riffles. The reach length in any of the locations in the Uma-oya and Knuckles streams was not less than 38 m, and the stream wetted width: length ratio was at the minimum of 1:6, and extended up to 1:400 in fish depauperate situations. In an effort to understand the spatial and

temporal scale fish assemblage compositions in study sites, duplicate surveys were conducted, representing low-flow months of southwestern and northeastern monsoons.

February and July (2009) samplings were conducted by electrofishing using back-pack EFKO equipment with Honda aggregate; blocking nets were not used. Three fishing passes were conducted at an interval of 35 to 45 min, with a minimum of 30 min of standing time between passes. Bateman et al. (2005) proposed single-pass electrofishing as providing accurate estimate of population measures, such as length-frequency distribution of fish. Initial fish surveys were conducted across multiple habitats, using a minimum of two pools and two riffles sections to be included in a predetermined stream section (reach) (Table 3-1). Some of the past studies of Sri Lankan freshwater stream fish community composition and their persistence have been described as pool- and riffle-dwelling species (Moyle and Senanayake 1984; Wikramanayake and Moyle 1989), with different species composition.

September (2009) and January (2010) samplings were conducted by three methods of netting (i.e. one cast nets, two dip nets and one drag net), employing four to five investigators and collecting all trapped fish. In order to harmonize fishing efforts between sites, three upstream passes were conducted for three types of netting, for example; (1) cast net was employed in consecutive and non overlapping stream sections, approximately 4 m in length (= diameter of the cast net); (2) 2 dip nets were employed for stream sections in edges in a similar way approximately 50 cm in diameter; and (3) drag net (1 m in width and 6 m in length) was employed for the middle stream sections. The stretched mesh size was 10 mm in all nets. The electrofishing and net samplings were standardized as far as possible between sites by means of number of passes, time between consecutive passes, and unit area. During net sampling, all sampling reaches were isolated at the top and bottom of each reach by stop nets (10 mm stretched mesh size), to prevent fish movement in or out of the area during sampling. In a few

occasions, natural boulder barriers provided adequate blockage. All samplings were conducted during the day time between 9:00 to 15:00 hrs.

Penczak and Glowacki (2008) studied electrofisher efficiency in stream habitats and have shown a high level of efficiency with less than 5% escapes. It was also reported that there has been no significant difference between pooled benthic and non-benthic fish species in the final catch. The cast net was more efficient than electrofishing in catching benthic *G. ceylonensis* in a long reach, which increased sampling efficiency considerably. All fish specimens were identified using morphological and meristic characteristics (Mendis and Fernando 1962; Pethiyagoda 1991; Pethiyagoda and Kottelat 2005; Silva et al. 2008), counted, and measured for total length (L) (± 0.1 cm), and weight (W) (± 0.01 g) on the site. Fish collected by multiple passes were pooled to represent the total assemblage for the given site, and given time.

3.3.2.1 Taxonomic discrepancies

Devario malabaricus is a widely distributed species in Sri Lanka (Pethiyagoda 1991), while it's closest relative *D. aequipinnatus* (McClelland, 1839) has been recorded in the Knuckles drainages (Pethiyagoda 2006). The *Danio* spp. has been interchangeably identified as *D. malabaricus* (Jones 1938b; Silas 1957; Pethiyagoda 1991; Weliange et al. 2006), and *D. aequipinnatus* (Schiemer and Hofer 1983; Schut et al. 1984; De Silva and Somarathna 1994), as distinct species (Graser et al. 1996), and declared to be synonymous (Meyer et al. 1993). Moyle and Senanayake (1984) and Wikramanayake and Moyle (1989) specified *D. malabaricus* as deep-bodied species that inhabits fast-flowing hill streams. Arunachalam (2000) reported homologous populations from the Western Ghats (India) streams as *D. aequipinnatus*. Detailed taxonomic studies on these "two forms" could not be found within the country. However, during the current fish survey some subtle morphological characters such as varied pattern of vertical elements (McClure and McCune 2003), and partly analogous dorsal body curveture to another

closest relative *D. pathirana* were observed in the Knuckles populations over Uma-oya (Figure 3-2). For the purpose of this chapter, the nomenclature of *D. malabaricus* (Jerdon 1849) will be used, in order to be consistent with the IUCN (2010), and the FishBase database (www.fishbase.org).

Despite broad geographic distribution of *G. ceylonensis* (Jones 1938a), Pethiyagoda (1991) reported a sympatric occurrence of *G. phillipsi* (Deraniyagala, 1933; Phillip's Garra) in hilly drainages of the Knuckles region (Jones 1938a). *Garra phillipsi* has shown almost no variation in taxonomical significant characters, and was once defined as a sub species of *G. ceylonensis* (Pethiyagoda 1991). We have initiated a genomic assessment of species differences for morphologically cryptic *Garra* specimens from prospective localities for possible sympatric occurrence. For the purpose of this chapter, all specimens of the genus *Garra* will be identified as *G. ceylonensis* (Bleeker 1863).

3.3.3 Sample analysis

All fish were maintained in well aerated water after capture. After measurements, all immature *G. ceylonensis*, *R. daniconius*, and *D. malabaricus* and all other species were released just below the sampling reach. To calculate fish species per unit area, total numbers were divided by the surface area (m^2) of each study reach, calculated as the length of the reach multiplied by the mean wetted-width, based on measurements taken at 5-m intervals. Water samples were taken (January 2010) in pre-cleaned plastic containers, and transported on ice, and analyzed in the Ecology and Environmental Biology laboratory of the Institute of Fundamental Studies, Kandy, Sri Lanka, for major water quality parameters using standard methods (APHA 2005). Electrical conductivity, temperature, and pH of surface water were measured *in situ*. The hourly temperature profiles in study streams were measured in selected locations by placing HOBO® water temp proV2 data loggers.

3.3.4 Data assessment and statistical analysis

Fish species diversity was measured in terms of evenness and richness using Shannon measure of diversity (Dodds 2002). Shannon index is expressed as: $H' = - \sum p_j \ln p_j$ where, in an assemblage of species, the $\sum$ represents the proportion of $_j$th individual species (p_j) summed to the total number of species (S). The evenness is E = H'/lnS where, E is the evenness, and S is the number of species. Since the present study focused on two streams in different geographies, the coefficient of biogeographic resemblance (CBR) was calculated; Adite and Thielen (1995) used the following formula; This coefficient was defined as follows: $CBR = 2C/(N_1 + N_2)$ where, C is the number of species common to two streams (localities), N_1 and N_2 the respective number of species in the stream in question; CBR ranges from 0 (no species in common) to 1 (both the same number and kinds of species shared). Fish species dominance was calculated for most abundant pair of species (*G. ceylonensis* and *D. malabaricus*) using: $C_1 = \sum(ni/N)^2$ where, ni is the number of individuals of two species and N is the total number of species collected. The values range from 0 to 1, the lower values show that the dominance of two species is shared with more species in the population, whereas the values closer to 1 illustrate that the dominance is shared by a fewer number of species. The value equals to 1 illustrates that the population is completely represented by *G. ceylonensis* and *D. malabaricus*.

Data were presented as either mean ± standard deviation (SD) or quantitative descriptors (%). Basic descriptive statistics on primary data were obtained using Minitab® version 16. Means of differences were tested using 1-way ANOVA, and consistencies in differences were identified by Kruskal-Wallis and followed by *post hoc* Dunn's test. The existence of correlations between fish density and stream gradient was tested by Pearson's correlation procedure. Qualitative assessments were made where appropriate. A canonical correspondence analysis (CCA) correlation plot (by XLSTAT® version 2010.3.05 for Windows XP) was performed on water quality variables to facilitate detection of any relationship with fish density, and diversity

parameters in selected sites. Statistical analyses were performed using Minitab® version 16 for Windows (Minitab, Inc.), and alpha levels were set at 0.05.

3.4 Results

3.4.1 Fish speciation in the Uma-oya

In total, 6,767 fish were captured, and measured in the Uma-oya. The total catch (%) of *D. malabaricus* was significantly ($p < 0.05$) improved by net sampling compared to electrofishing, but both methods achieved similar measures of relative abundance for *G. ceylonensis* (Figure 3-3); there were insufficient data to compare with other species. Fourteen species were identified from the Uma-oya, and tributary streams, and cyprinids represented over 46% of the catch; they had previously been shown to be the most abundant fish species (Silva 1993; Jinadasa et al. 2003; De Silva et al. 2006). Initial surveys in the uppermost streams in the higher ranges in the Uma-oya catchment in Nuwara Eliya (1,865 m above MSL) found no fishes and further studies were restricted to downstream locations. The highest elevation site at Perawella (1,187 m above MSL) contained only two fish species: *G. ceylonensis* (72.4%) and *Lepidocephalichthys thermalis* (Cobitidae; 27.6%). Between four and seven species were collected at all other sites in the Uma-oya (Table 3-2), with the exception of Puhulpola, which contained nine species. All species represented <10% of the catch, except for a few dominant species at each site, and the dominant species varied between sites. In the middle catchments, regardless of capture method, more than 80% of individuals were either a combination of *G. ceylonensis* and *D. malabaricus* or *G. ceylonensis* and *R. daniconius* in nine out of eleven surveys. Irrespective of the stream topography (i.e. riffle, pool, run or mix), season of sampling and fishing method, all of the sites in the Uma-oya were dominated by *G. ceylonensis*, except Girambe (July and January), Puhulpola (September and January), and Mathatilla (January), and *G. ceylonensis* was completely absent in the Kottegoda site. Alternatively, *G. ceylonensis* had

second dominance to *D. malabaricus* in five occasions of their co-occurrence. *Garra ceylonensis* and *D. malabaricus* combination (co-occurrence) accounted over 65.6 through 96.7% of catches at any of the sites. Only *G. ceylonensis* in Erabadda had ~76% of abundance that meets the abundance scale (76–100%) by a single species, as proposed by Jadhav et al. (2011).

The upper Mahaweli streams were also dominated by either *R. daniconius* (17.8 and 42.8% of the catch at Diganathenna and Kottegoda locations) or *G. ceylonensis* (50.4 and 75.6% of the catch at Diganathenna and Erabadda). The Erabadda site was dominated by *G. ceylonensis* and *R. daniconius* at all times (February, July, September and January), representing between 56 and 100% of the catch. *Devario malabaricus* dominated at higher elevation sites at Girambe, Mathatilla and Puhulpola, but sites high in *D. malabaricus* were never high in abundance of *R. daniconius*; at Erabadda and Kottegoda where *R. daniconius* was found, not a single specimen of *D. malabaricus* was found. However, in sites where co-occurrences were observed (Puhulpola, Mathatilla and Diganathenna), *R. daniconius* was always at a lower abundance (Table 3-2).

Though the *Puntius* genus accounts for more than twenty Sri Lankan cyprinid species (Froese and Pauly 2010), there was limited representation in the Uma-oya (Table 3-2). Out of five *Puntius* spp. reported for most dominance in highland streams (Kortmulder 1982), *Puntius bimaculatus* was recorded widely in the Uma-oya, in excess of 5%, and only one specimen each of *P. dorsalis* and *P. sarana* was encountered, even though it has been reported as a broadly distributed species across different elevations (De Silva et al. 2006).

Other abundant species (>5%) included *Lepidocephalichthys thermalis*, *Channa gachua*, *Poecillia reticulata* (Peters 1859), and *Oreochromis niloticus*, and a few individuals were collected of *Xiphophorus hellerii*, *Tor khudree*, and one specimen each of *Anguilla* sp., and *Neomacheilus notostigma*. Nile tilapia (*O. niloticus*) were collected in one of the tributary drainages in Uma-oya (Kottegoda–N06°57'41.7" E80°56'09.7" 963 m above MSL) (5/100 m^2), along with *P. bimaculatus* (13/100 m^2) and striped rasbora (*R. daniconius*) (16/100 m^2) in

January 2010; this was the only site, where *G. ceylonensis* were completely absent. Twenty six individuals of *O. niloticus* in a total length (range) 8.5 to 28.3 cm, and weight of 11.25 to over 200 g were caught in one collection. This is the first record of the existence of *O. niloticus* in the Uma-oya.

Other than species composition, the population structure of fish exhibited some striking differences among sampling sites. In general, length-frequency data of *G. ceylonensis* in sites, where they were present showed clear population structure; left-ward shifting recruitment clusters, and right-ward shifting aged-clusters. The skewness in length-frequency probability plots of *G. ceylonensis* in Medawela (Uma-oya) were unimodal/ bimodal, and normally distributed ($p < 0.05$), while in Puhulpola, the population structure was staggered with prominent loss of individuals in some size-classes (Figure 3-4).

3.4.2 Fish speciation in the Amban-ganga catchment (Knuckles streams)

Cyprinid species made up 68.6% (11 of 16) of species found in the Knuckles streams out of 1,063 fish specimens collected (Table 3-2), with six families represented. *Garra ceylonensis* was the dominant species (84.5%) in Etanwala (Theligam-oya upstream). In the rest of the three sites, *D. malabaricus* was dominant and *R. daniconius* the second most dominant when all three species were present. More than 80% of individuals were from three species, with *G. ceylonensis*, *D. malabaricus*, and *R. daniconius* representing 880 individuals, out of total 1,063 (82.8%); the remaining twelve species were rare. In Rambukoluwa (Kalu-ganga stream), *R. daniconius* and *P. bimaculatus* occurred with similar abundance (16.6 and 17.2%, respectively). Cyprinids such as *P. martenstyni* and *Tor khudree* represented over 10% of individuals in two locations in the Knuckles streams. Similar to the Uma-oya, *R. daniconius* was completely absent from Etanwala and Illukkumbura sites (Theligam-oya upstream and downstream locations, respectively) while slight to strong inhibition by *D. malabaricus* was observed in Rambukoluwa (Kalu-ganga) and Ranamure-oya sites (Table 3-2).

Neomacheilus notostigma (Balitoridae) and *Lepidocephalichthys jonklaasi* (Cobitiidae) collectively represented 6% in the Ranamure-oya site. Only two specimens of *L. jonklaasi* were collected along the reach, which had the second lowest relative abundance at 0.8%. Several other fish species who had less than 5% abundance were *Puntius singhala*, *P. kamalika*, *P. dorsalis*, *P. srilankensis*, *D. pathirana* (family: Cyprinidae), *Belontia signata* (Osphronemidae), *Channa orientalis* (Channidae), and *Clarias brachysoma* (Clariidae). There were nine endemic freshwater fish species found in the Knuckles streams, out of twelve endemics recorded from the Mahaweli River basin by Silva (1993).

3.4.3 Fish faunal similarity and endemism

The highest Shannon fish diversity (2.01) was present in Rambukoluwa (Kalu-ganga), and the lowest (0.43) in Etanwala (Theligam-oya upstream) in the Amban-ganga catchment. The ichthyofaunal similarity between the two catchments was 39% (Table 3-3), and fish species diversity was negatively correlated with stream elevation gradients (Pearson's correlation -0.630, $p = 0.028$).

All sites in the Knuckles streams were coordinated with diversity rather than density indices. Fish density estimates did not show linear relationships with environmental variables (e.g. nitrate, phosphate, sulphate, chlorophyll-*a* content) ($p = 0.189$) (Figure 3-5). However, there were significant pair-wise correlations of phosphate (Pearson's correlation 0.851, $p < 0.001$), and nitrate (Pearson's correlation 0.662, $p = 0.019$) with *G. ceylonensis* density, and overall fish density with phosphates (Pearson's correlation 0.883, $p < 0.001$), and nitrates (Pearson's correlation 0.783, $p = 0.003$). Also, there was a positive correlation of nitrate concentrations in study sites and *D. malabaricus* density (Pearson's correlation 0.617, $p = 0.033$).

Endemism is highest in the Amban-ganga (reference) catchment in the Knuckles area compared to the agricultural (Uma-oya) catchment (Table 3-3). Kalu-ganga in the Knuckles

showed highest endemism (61.5%) where, seven out of eleven species were endemic to the country. In the Uma-oya, only *G. ceylonensis* and *N. notostigma* were found to be endemic; the latter was a single specimen (Kottegoda site). *Puntius bimaculatus* (Bleeker 1863) was one of the common species found in both geographies; relative abundance varied from 0.8 to 34.6% in the Uma-oya stream and 1.1 to 17.2% in the Knuckles streams (Table 3-3).

One of the most intriguing observations of this study was that there seemed to be small-scale inhibition between the two fish species– striped rasbora (*R. daniconius*), and giant danio (*D. malabaricus*). Of the eight sites in the Uma-oya stream, *R. daniconius* was found only in five sites and, except for Puhulpola, the other four locations can be categorically identified as tributary streams from various altitudes (Figure 3-1); attempts to find *R. daniconius* in extended stream sections outside the pre-determined sampling reaches were unsuccessful. There were comparable abundance of *R. daniconius* in Erabadda site (1,106 m above MSL) in February (2009), July (2009), September (2009) through January (2010), ranging from 7 to 24 individuals per 100 m^2.

In the Uma-oya catchment, it is increasingly common to observe thick stands of giant cane (*Arundo donax*), either planted or invaded in stream banks. The relative contribution of this vegetation type in stream banks of eight study sites was in a range from none (i.e. zero percent, where dominant vegetation type other than *A. donax*) to 100% of *A. donax* (Table 3-1). There was a negative relationship (Herrera and Dudley 2003) between percent *A. donax* stand in the riparian area, and the abundance of *R. daniconius* (Figure 3-1), although the correlation was statistically non significant (Pearson's correlation -0.541, $p = 0.165$). However, quantitative observations showed that at sites ($n = 4$) where *A. donax* stand was 70 to 100%, had a lesser abundance (0–1.7%) of *R. daniconius*. Alternatively, in sites ($n = 3$) where *A. donax* stand was 0 to 40%, had a higher relative abundance of *R. daniconius* in a range from 11.6 to 42.7%; the

only exception was at Mathatilla where lesser abundance of *R. daniconius* (4.1%) was observed under lesser riparian stand of *A. donax* (40%).

3.5 Discussion

In total, 23 fish species were categorically identified in the Uma-oya and Knuckles streams. Sri Lankan freshwater fish species are predominantly zooplanktivorous, insectivorous, detritivorous, and macrophyte feeders, relying on vision for feeding (Weliange et al. 2006). Sampling may have underestimated carnivorous predatory species that are more active at night (Ghosh et al. 1990; Bhat 2003). Although *Clarias teysmanni*, *Ompok bimaculatus*, *Channa striata*, *C. punctata*, *C. marulius*, and *Anabas testudineus* have been reported to be present in the Mahaweli River basin, only *Clarias brachysoma, Channa gachua*, and *C. orientalis* were encountered (Appendix 1). *Channa gachua* is one of the secondary consumers in Sri Lankan freshwater fishes (De Silva 1991a), and they may have contributed to the fish structure in some of the locations in the Uma-oya. Mirandu et al. (2008) reported the presence of *C. orientalis* in the Knuckles streams, which has been restricted to the southwestern ichthyological province.

No native species were found at the highest elevation, which agrees with with Costa (1974) that highland plateau streams at an elevation of approximately 2,200 m are sparsely colonized by rainbow trout (*Oncorhynchus mykiss*) that were introduced to Sri Lanka in 1889. The highest site with fish (1,187 m) contained only *G. ceylonensis* and *Lepidocephalichthys thermalis*, which are both benthic dwellers, adapted for periodic torrential conditions (Bond 1996) with dorsoventrally compressed bodies with flattened heads, broad pectoral fins, and reduced or absent gas bladders (Wikramanayake 1990). Previous studies have concluded that the highland plateau in Sri Lanka is dominated by either *G. ceylonensis* and *L. thermalis* (Jinadasa et al. 2003) or *R. daniconius* and *N. notostigma* (Moyle and Senanayake 1984). Silva (1993) reported the occurrence of *L. jonklaasi* on the southern slopes of the Knuckles range although

Pethiyagoda (1991), and Ott and Ende (2004) have reported its type locality in southwestern parts of Sri Lanka.

According to the ichthyofaunal compilation by Silva (1993), 44 species have been recorded in the Mahaweli River basin out of 58 indigenous and endemic fish species in Sri Lanka, with *G. ceylonensis*, *R. daniconius*, and *D. aequipinnatus* most common. Both stream catchments showed these species to be dominant, consistent with Starmühlner (1984), who reported that upper-course fish community between 500 and 1,500 m were dominated by populations of *D. malabaricus*, *R. daniconius*, *P. bimaculatus*, *L. thermalis*, and *N. notostigma* under low flow velocity regimes of 0 to 75 cm sec^{-1}. Starmühlner (1984) also found that *G. ceylonensis* dominated at current velocities from 30 to above 100 cm sec^{-1}, implying that *G. ceylonensis* prefers high flow area. *Garra ceylonensis* is widely distributed in Sri Lanka across streams below 100 to 1,500 m above MSL elevations (Jinadasa et al. 2003; Weliange 2007; Starmühlner 1984), and *N. notostigma* is a common dispersant in mid to high elevations (500 to 1,500 m above MSL) under low flow velocity regimes of 0 to 75 cm sec^{-1} (Starmühlner 1984). Although several authors (Jinadasa et al. 2003; Silva 1993) identified *P. bimaculatus* as endemic to Sri Lanka, recent records (Froese and Pauly 2010) suggest this may be a common dispersant from the Indian mainland. The diversity and composition of fish assemblages follow a gradient of longitudinal changes in habitat of a river/ stream and fish populations are related to stream size and order (Vannote et al. 1980). Generally, there is a progressive increase in ichthyomass downstream, thus relatively a small ichthyomass could be expected in small streams (Edds 1993).

The abundance of *D. malabaricus* in either the first or second rank in highland streams has not been reported in other catchments (Jinadasa et al. 2003). Similarly, Arunachalam (2000) recorded *D. aequipinnatus* (= *D. malabaricus*; Pethiyagoda 1991), *R. daniconius* and *G. mullya* were most abundant in all streams within 600 and 800 m elevations in the Western Ghats in

India. The similarities of fish communities in Sri Lanka and Western Ghats have also been reported by Kortmulder et al. (1990). If there really is spatial competition between these species, as previously suggested by Moyle and Senanayake (1984), this could partly explain the absence or near-absence of *R. daniconius* from some of the locations in the Uma-oya and the Knuckles streams with high densities of *D. malabaricus*. It has been suggested that *R. daniconius* and *D. malabaricus* are mutually incompatible for niche utilization for food resources (Moyle and Senanayake 1984; Schut et al. 1984), although there were several sites in this study, where they were mutually abundant: Rambukoluwa (217 m above MSL); February, 2009 in Ranamure-oya (339 m above MSL), and Diganathenna in the Uma-oya (1,058 m above MSL).

The limited fish faunal assemblages in the Uma-oya are ecologically significant as only single species of *Garra*, *Devario* and *Rasbora* are represented in Sri Lanka, and 14, 6 and 14 species are reported in these genera in India, respectively (Wikramanayake 1990). Of 17 member species of the genus *Garra* reported on the African continent, 11 species have been reported in Ethiopia alone (Stiassny and Getahun 2007). The significant abundance of *D. malabaricus* in the Mahaweli River basin (15.5–61% in the Knuckles streams and 26.9–70.1% in the Uma-oya stream), in contrast to 0.06% in the Walawa River basin (Jinadasa et al. 2003), suggests that they may also be useful as an environmental sentinel. According to De Silva et al. (1980), *R. daniconius* and *Devario* spp. (i.e. *D. aequipinnatus*) were recorded to be amongst the co-habiting species in Sri Lankan streams. However, Moyle and Senanayake (1984) speculated limited co-occurrence of *R. daniconius* with *D. malabaricus* due to competition for niche dimensions. The lesser abundance of *G. ceylonensis* in the Knuckles streams may be site-specific, where abundance of food resources such as algal biomass is limited, due to aerial forest cover (De Silva 1991b).

One of the interesting observations of this survey was the occurrence of *Devario pathirana* and *P. kamalika* in the Kalu-ganga (Rambukoluwa), which have been reported to be

found from the southwestern ichthyological province before (Pethiyagoda 1991). These species have not been recorded in the ichthyofaunal compilation of the Mahaweli River basin by Silva (1993). In fact, Silva's (1993) freshwater fish compilation in the Mahaweli ichthyological province did not record the presence of *Puntius* (= *Barbus*) *amphibius*, which was subsequently named as *P. kamalika* (Silva et al. 2008) (Appendix 1).

Stream types in Sri Lanka can be differentiated into rocky hill (Perawella– Uma-oya stream; Etanwala and Ranamuregama– Knuckles streams) *versus* winding hill (all others), into wet zone and dry zone streams, and into high elevation and low elevation. Schut et al. (1984) found that *R. daniconius* distribution was restricted to winding hill streams while *D. malabaricus* to rocky hill streams. Weliange (2007) reported that *R. daniconius* is one of the species commonly found in the wet zone and the dry zone in Sri Lanka (below 200 m above MSL). Along a longitudinal gradient in the upper Walawe River basin from an elevation of 200 m up to 2,000 m above MSL, Jinadasa et al. (2003) recorded that the rank abundance at lower elevations was in the order of *R. daniconius>P. bimaculatus>G. ceylonensis*, whereas the abundance was in the order of *G. ceylonensis>Schistura notostigma>L. thermalis* above 600 m MSL. Therefore, *R. daniconius* in principle may be a low elevation species, but Jinadasa et al. (2003) did not observe a dominant distribution of *D. malabaricus* in higher elevations in Sri Lanka, as observed in our study. The co-occurrence and positive association of fish species in assemblages is a subject of extensive debate in tropical streams (Bhat and Magurran 2007).

Distributions are commonly assigned to ichthyofaunal provinces, which include endemic species, which are "native to, and restricted to that system" (Senanayake and Moyle 1982; Lowe-McConnell 1993), and Sri Lanka has 14.2% proportion of endemic freshwater finfish species (De Silva et al. 2007). The Knuckles streams were host for several of the highly endangered or vulnerable fish species, such as *D. pathirana*, *L. jonklaasi*, *P. srilankensis*, and *P. martenstyni*. Pethiyagoda (2006) listed 29 endemic freshwater fish species as threatened, and 10

as highly threatened, in the context of the national fish conservation agenda. Several of these species are confined to the Knuckles streams, including *P. srilankensis* (Pethiyagoda and Kottelat 2005), *G. phillipsi*, and *D. aequipinnatus* (Pethiyagoda 1991), which are listed as data deficient (DD) in the IUCN Red List (IUCN 2010), due to incomplete life history status.

Data on biodiversity in Sri Lanka is not complete; several cyprinids have recently been added to freshwater fishes (Meegaskumbura et al. 2008; Pethiyagoda et al. 2008a), and one species has been rapidly declining (*Macrognathus pentophthalmos* (Teleostei: Mastacembelidae) (Pethiyagoda et al. 2008b). *Puntius singhala* (Duncker 1912), and *P. kamalika* are endemic and have been recently identified elsewhere in Sri Lanka (Silva et al. 2008), and were probably previously misidentified as *P. filamentosus* and *P. amphibius*, respectively (Pethiyagoda and Kottelat 2005; Gunawickrama 2008). Some of the species known to occur in the southwestern ichthyological province (type locality) were identified in the Mahaweli ichthyological province, which include *C. brachysoma*, *D. pathirana*, and *P. kamalika* (Pethiyagoda 1991; Silva et al. 2008). This implies that conservation strategies should expand to a wider geographical range, than earlier assumed.

The present study revealed that the density of *G. ceylonensis* was positively associated with phosphate and nitrate levels, and more interestingly *D. malabaricus* with nitrate levels at the catchment level. Dejenie et al. (2009) similarly found that *Garra* biomass was positively related to nutrients in reservoirs. *Garra* was positively associated with *Devario* in the Uma-oya stream, suggesting that maximum resource utilization may be at the expense of other species. *Puntius dorsalis* was depauperate in the middle catchment (900 to 1,200 m above MSL) of the Uma-oya. De Silva et al. (2006) observed that *P. bimaculatus* was positively correlated, and *P. dorsalis* was negatively correlated with conductivity and phosphate. The intensive year-round agricultural practices over the past several decades may have placed stress on *P. dorsalis*

populations in the Uma-oya. Eutrophication presumably increases the algae and diatoms (De Silva et al. 2006), that *P. bimaculatus* feeds on (Pethiyagoda 1991).

In order to relate change in species diversity to alteration in water physico-chemical variables requires a considerable database for acceptable correlations (Martin-Smith and Laird 1998). Obviously, low oxygen may have least impact on *R. daniconius* because this species can exploit water-air inter-phase, and gulp air while feeding (Martin-Smith and Laird 1998). Bhat and Maguran (2007) stated that habitats that are altered or disturbed by human activities can result in deviations from the co-occurrence patterns found in undisturbed localities. Since all sampling sites with low abundance of *R. daniconius* are located in the stem stream of the Uma-oya, extensive pollutant impacts could be anticipated. Habitat changes may also be playing a role– giant cane (*Arundo donax*) originated in the Asia and Mediterranean countries, has become widely established in the Uma-oya catchment (Boose and Holt 1999). *Arundo* stands may impact on *R. daniconius* existence, whose diets are largely composed of allochthonous insects sourced from riparian vegetation (Starmühlner 1984). Prem Kumar and John (2007) have shown that *R. daniconius* prefers terrestrial drift food, mainly feeding on hymenopterans and coleopterans.

There was a consistently higher number of *Puntius* species in a single location in Kaluganga stream (Knuckles) with a total species composition of six as compared to three (besides *P. bimaculatus*, only a single specimen each of *P. dorsalis*, and *P. sarana* were found) across eight locations in the middle catchment (altitude, 900 to 1,200 m above MSL) of the Uma-oya. Jinadasa et al. (2003) recorded eight *Puntius* species across the Walawa River basin, while Fernando et al. (2000) recorded eleven species across four major river basins in Sri Lanka, with co-associations on various aspects (Schut 1984).

3.5.1 Potential impact by exotic fish species

It is well known that anthropogenic impact favors exotic invasions (Kennard et al. 2005; Casatti et al. 2006). No exotic species were found in the Knuckles reference area, but *O.*

niloticus (Linnaeus 1758) (family Cichlidae), *P. reticulata* (Peters 1859) and *X. helleri* were found in the agricultural areas (Uma-oya). Collares-Pereira and Cowx (2004) reported species introductions as the leading threat to freshwater fishes in the world, and their presence is regarded as a serious biotic pollutant (Harris 1995). In Sri Lanka, the out-competing of indigenous stream fishes by exotics (i.e. especially tilapian fishes) has not been reported in the literature (Fernando et al. 2000; Wijeyaratne and Perera 2001; Amarasinghe et al. 2004; Dudgeon and Smith 2006), but negative concerns have mounted on exotics with limited evidence (Pethiyagoda 1994; Weyl 1994; Pethiyagoda 2006).

The introduction of *O. niloticus* was intensified in mid-1980 due to its fast growth and lower trophic position (Costa-Pierce 2003), and its use by fisheries biologists and managers for aquaculture and fisheries development. Reservoir fishing of exotic species is one of the important food fish exploitation ventures in Sri Lanka (Fernando 1984), and these species disperse because of unintentional releases from aquaculture and flooding (Esselman 2009). Most of the endangered and vulnerable freshwater fish species (73%) in Sri Lanka occur only in lotic habitats associated with forested streams in higher altitudes, and the lower thermal tolerance of *O. niloticus* compared to *O. mossambicus* (Philippart and Ruwet 1982) suggests it is a threat to hillside stream fish communities. Fernando et al. (2000) caught sixty six (66) individuals of *O. mossambicus* during 278 fish collections in the Kelani River basin; *Oreochromis niloticus* is a food fish species introduced into inland reservoirs, and can be invaded or intentionally or unintentionally introduced into tributary rivers and streams subsequently (Fernando et al. 2000).

Poecillia reticulata (Peters 1859) and *X. hellerii* are exotic fish introductions from the aquarium industry (Wijesekara and Yakupitiyage 2001). *Xiphophorus hellerii* was found in two locations, whereas *P. reticulata* was found in five locations, and exceeded 5% in Girambe in the Uma-oya. The presence of these exotics should be viewed with caution since Goren and Galil (2005) reported examples of non-native poeciliids out-competing native species, and causing the

extirpation of *G. rufa* from the Qishon River basin, and *G. ghoerensis* from the southern Dead Sea in Israel. Members of Poeciliidae prey upon indigenous and endemic fish larvae, and post-larvae, which have been deprived of sheltering structures. Casatti et al. (2006) observed *P. reticulata* abundance over 50% in degraded habitats in Brazilian streams. Orrego et al. (2009) reported the occurrence of *Gambusia affinis* (Family: Poeciliidae) in association with pollution in Chilean rivers.

3.5.2 Impact of exploitation

Local people exploit some populations as a protein source in the Uma-oya and the Knuckles tributaries for large "food-size" individuals, such as *G. ceylonensis*, *Tor khudree*, and *C. gachua* (Jayakody A. Sumith, pers. observ.). The Puhulpola site seems to be a fishery hotspot for local subsistence fishermen using gill nets and cast nets, which result in discrete size classes. Highly crafted fish traps made of twigs called *"kemana"* are used as fixed contrivances in the form of funnel shapes at the upstream drainages to catch ascending fish during spawning times (see Figure 3-6). This type of fishing method has been possible due to reported upstream migration of *Garra* spp. in small rocky hill streams for spawning, with the onset of heavy rains (Pethiyagoda 1991; Silva 1993).

In conclusion, we selected an agriculturally exploited catchment in the Uma-oya, and a least-exploited catchment in the Knuckles forested area for baseline data. The Uma-oya catchment shows characteristics commonly observed in extensive agricultural exploitation, such as impaired water quality and altered riparian vegetation. Eutrophication by agricultural activities is presumably one of a number of other contributors for increased abundance of fish species, such as *G. ceylonensis*, and *D. malabaricus*. Therefore, only a strong philosophical decision would dictate whether responses, such as eutrophication are bad or unacceptable (Munkittrick and Van Der Kraak 1999). However, in taking decisions in such cases, we see

value in taking ecosystem into consideration to predict likely responses to any management action, whether those decisions fulfill "societal values" or "ecosystem objectives".

The most abundant fish species in the two regions were *G. ceylonensis*, *D. malabaricus*, and *R. daniconius*, although their relative abundance differed between sites. The Knuckles streams showed no exotic species, and a higher level of endemism. It appears that extensive agriculture activities may induce fish species "homogenization" (favoring more abundance of certain groups of indigenous species over others), but inadequate data on "historical" status in specific ecosystems prevent making strong conclusions.

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Appendix 1. List of native freshwater and brackish water fish species in Sri Lanka, and the status of geographic similarity with the Western Ghats (India).

Order	Family	Species*	Existence in the Mahaweli River basin (Sri Lanka)**	Existence in the Western Ghats (India)***
Elopiformes	Megalopidae	*Megalops cyprinoids* (Broussonet)	×	√
Anguilliformes	Anguillidae	*Anguilla nebulosa* (McClelland)	√	×
		A. bicolour (McClelland)	√	√
		A. bengalensis (Gray)	×	√
Clupeiformes	Clupeidae	*Ehirava fluviatilis* (Deraniyagala)	√	×
Cypriniformes	Cyprinidae	*Amblypharyngodon melettinus* (Valenciennes)	√	×
		A. grandisquammis (Jordan and Starks 1917)	× E	×
		Chela laubuca (Hamilton-Buchanan)	√	√
		C. ceylonensis (Hamilton-Buchanan 1821)	× E	×
		Devario malabaricus (Jerdon)	√	√
		D. pathirana (Kottelat and Pethiyagoda 1990)	√ E (this study)	×
		D. aequipinnatus (McClelland 1839)	√	√
		Esomus thermoicos (Valenciennes)	× E	×
		E. danricus (Hamilton-Buchanan)	√	√
		E. lineatus (Ahl)	×	×
		Garra ceylonensis (Bleeker)	√ E	×
		G. phillipsi (Deraniyagala 1933)	√ E	×
		Labeo dussumieri (Valenciennes)	√	√
		L. fisheri (Jordan and Starks 1917)	√ E	×
		L. Lankae (Deraniyagala)	× E	×
		L. porcellus (Heckel 1838)	×	√
		L. rohita (Hamilton-Buchanan)	×	√
		Laubuca insularis (Pethiyagoda, Kottalat, Silva, Maduwage and Meegaskumbura 2008)	× E	×

		L. lankensis (Pethiyagoda, Kottalat, Silva, Maduwage and Meegaskumbura 2008)	× E	×
		L. ruhuna (Pethiyagoda, Kottalat, Silva, Maduwage and Meegaskumbura 2008)	× E	×
		L. varuna (Pethiyagoda, Kottalat, Silva, Maduwage and Meegaskumbura 2008)	× E	×
		Puntius asoka (Kottelat and Pethiyagoda 1989)	× E	×
		P. bandula (Kottelat and Pethiyagoda 1991)	× E	×
		P. amphibius (Valenciennes)	×	√
		P. bimaculatus (Bleeker)	√	√
		P. chola (Hamilton-Buchanan)	√	√
		P. cumingii (Günther)	√ E	×
		P. dorsalis (Jerdon)	√	√
		P. martenstyni (Kottelat and Pethiyagoda 1991)	× E	×
		P. nigrofasciatus (Günther)	√ E	×
		P. pleurotaenia (Bleeker)	×	√
		P. sarana (Hamilton-Buchanan)	√	√
		P. singhala (Duncker)	√ E	×
		P. srilankensis (Senanayake 1985)	√ E	×
		P. titteya (Deraniyagala)	√ E	×
		P. vittatus (Day)	×	√
		P. ticto (Hamilton-Buchanan)	√	√
		P. kamalika (Silva, Maduwage and Pethiyagoda)	√ E (this study)	×
		P. reval (Meegaskumbura, Silva, Maduwage and Pethiyagoda)	× E	×
		P. punctatus (Day)	×	×
		P. kelumi (Pethiyagoda, Silva, Maduwage and Meegaskumbura)	× E	×
		Rasbora caverii (Jerdon)	×	√
		R. daniconius (Hamilton-Buchanan)	√	√
		R. wilpita (Kottelat and Pethiyagoda 1991)	× E	×

		R. vaterifloris (Deraniyagala)	√ E	×
		R. armitagei (Gabadage and Karunaratna 1994)	× E	×
		R. naggsi (Manamendra-Arachchi and Perera 1993)	× E	×
		Tor khudree (Sykes)	√	√
		Horadandia atukorali (Deraniyagala)	×	×
	Balitoridae	*Acanthocobitis urophthalmus* (Günther)	× E	×
		Nemacheilus notostigma (Bleeker)	√ E	×
	Cobitidae	*Lepidocephalichthys jonklaasi* (Deraniyagala 1956)	√ E	×
		L. thermalis (Valenciennes)	√	√
Siluriformes	Bagridae	*Mystus gulio* (Hamilton-Buchanan)	×	√
		M. cavasius (Hamilton-Buchanan)	×	√
		M. vittatus (Bloch)	√	√
		M. keletius (Valenciennes)	√	√
		M. ankutta (Pethiyagoda, Silva and Maduwage 2008)	× E	×
	Clariidae	*Clarias brachysoma* (Günther)	√ E (this study)	×
		C. nebulosus (Deraniyagala)	× E	×
		C. *teysmanni* (Bleeker)	√	×
	Heteropneustidae	*Heteropneustes fossilis* (Bloch)	√	√
		H. microps (Günther 1864)	×	×
	Siluridae	*Ompok bimaculatus* (Bloch)	√	√
		Wallago attu (Bloch and Schneider)	√	√
Cyprinodontiformes	Aplocheilidae	*Aplocheilus dayi* (Steindachner)	×	×
		A. parvus (Raj)	×	×
		A. werneri (Meinken)	×	×
		A. blockii (Arnold)	×	√
		A. panchax (Hamilton-Buchanan)	×	√
		A. lineatus (Valenciennes)	×	√
Beloniformes	Adrianichthyidae	*Oryzias melastigma* (McClelland)	×	√
	Belonidae	*Xenentodon cancila* (Hamilton-Buchanan)	×	√
	Hemirhamphidae	*Hyporhamphus limbatus* (Valenciennes)	√	√

		Zenarchopterus dispar (Valenciennes)	×	×
	Mastacembelidae	*Macrognathus aral* (Bloch and Schneider 1801)	×	×
		M. pentophthalmos (Gronow, in Gray 1854)	×	√
		Mastacembelus armatus (La Cepūde 1803)	√	×
Sygnathiformes	Syngnathidae	*Microphis brachyurus* (Bleeker)	×	×
		M. ocellatus (Duncker)	×	×
Synbranchiformes	Synbranchidae	*Ophisternon bengalense* (McClelland)	×	×
		Monopterus desilvai (McClelland)	× E	×
Perciformes	Anabantidae	*Anabas testudineus* (Bloch)	√	√
	Cichlidae	*Etroplus maculatus* (Bloch)	√	√
		E. suratensis (Bloch)	√	√
	Channidae	*Channa ara* (Deraniyagala)	× E	×
		C. gachua (Hamilton-Buchanan)	√	×
		C. orientalis (Bloch and Schneider)	√	√
		C. punctata (Bloch)	√	√
		C. striata (Bloch)	√	√
		C. marulius (Hamilton-Buchanan)	√	√
		C. asiatica (Linnaeus)	×	×
	Monodactylidae	*Monodactylus kottelati* (Pethiyagoda 1991)	×	×
	Kuhliidae	*Kuhlia marginata* (Cuvier)	×	×
	Eleotridae	*Eleotris fusca* (Forster)	×	×
		Butis butis (Hamilton-Buchanan)	×	×
	Gobiidae	*Awaous melanocephalus* (Bleeker)	×	×
		Sicyopterus griseus (Day)	×	√
		S. halei (Day 1888)	× E	×
		Sicyopus jonklaasi (Klausewitz and Henrich 1986)	× E	×
		Stenogobius macropterus (Duncker)	× E	×
		Stiphodon martenstyni (Watson)	× E	×
		Schismatogobius deraniyagalai (Kottelat and Pethiyagoda 1989)	× E	×

		Glossogobius giuris (Hamilton-Buchanan)	√	√
	Osphronemidae	*Belontia signata* (Günther)	√ E	×
		Malpulutta kretseri (Deraniyagala)	× E	×
		Pseudosphromenus cupanus (Cuvier)	×	×
		Trichogaster pectoralis (Regan)	×	×
		Osphronemus goramy (Lacepede)	×	√
	Toxotidae	*Toxotes chatareus* (Hamilton-Buchanan)	×	×
Tetraodontiformes	Tetraodontidae	*Tetraodon fluviatilis* (Hamilton-Buchanan)	×	×
		T. nigroviridis (Marion de Procé)	×	×

(√) present; (×) absent; (E) endemic to Sri Lanka

* Pethiyagoda (1991); Silva (1993); Froese and Pauly (2010); Silva et al. (2010)

** Silva (1993)

*** Bhat (2004); Dahanukar et al. (2004); Shreekantha et al. (2007); Shahnawaz et al. (2010)

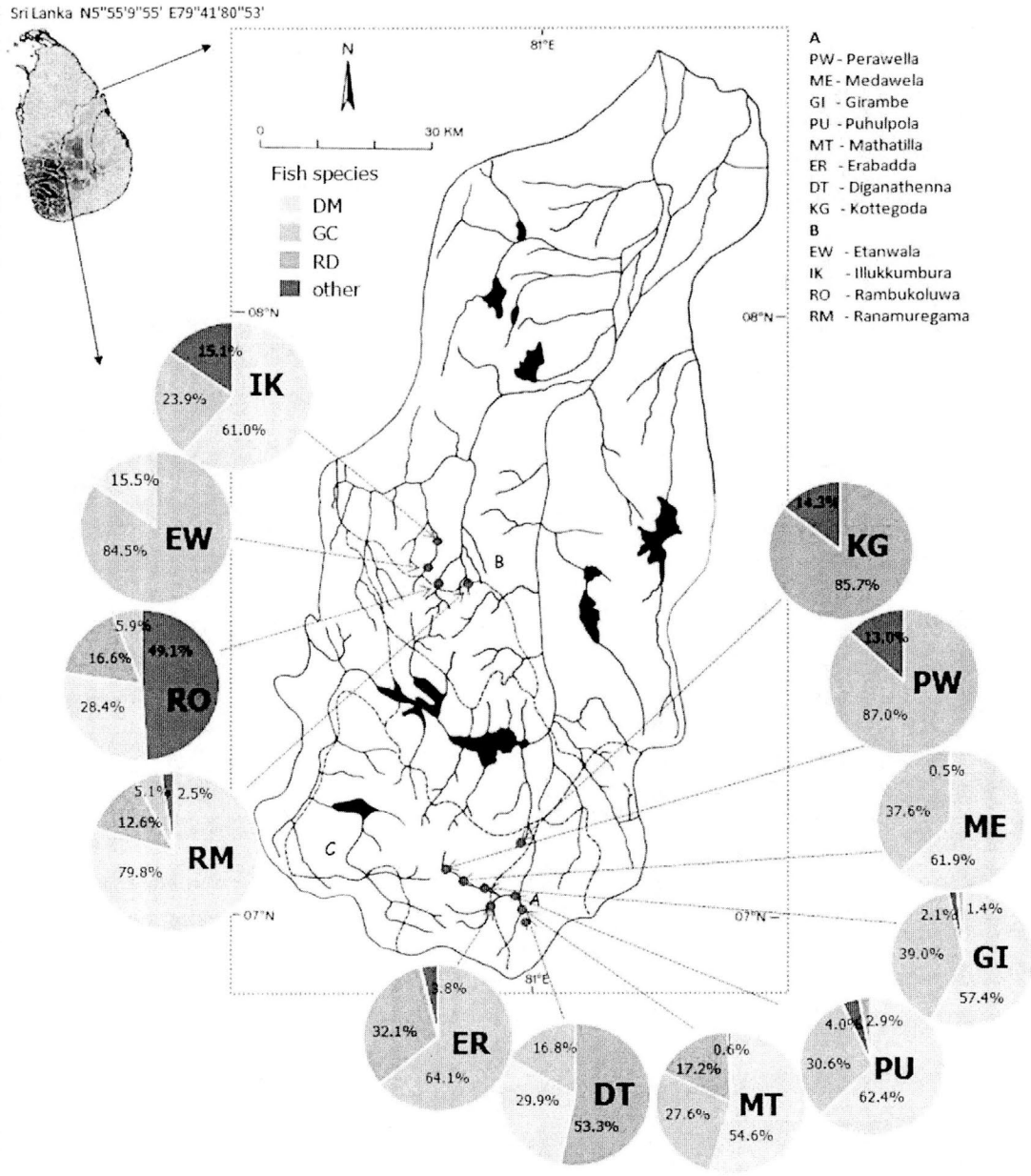


Figure 3-1. Map of major stream distribution and sampling locations in the Mahaweli River basin of Sri Lanka: (A) Location of the Uma-oya catchment and sampling sites; and (B) Location of the Amban-ganga catchment and sampling sites (Modified from Silva 1993). Sampling sites are shown by (●). Pie-charts show fish species composition (% by density) of most abundant species of *Garra ceylonensis* (GC), *Devario malabaricus* (DM) and *Rasbora daniconius* (RD) estimated in a single sampling event using composite nets in January, 2010. The composition of other species is as per Table 3-2.

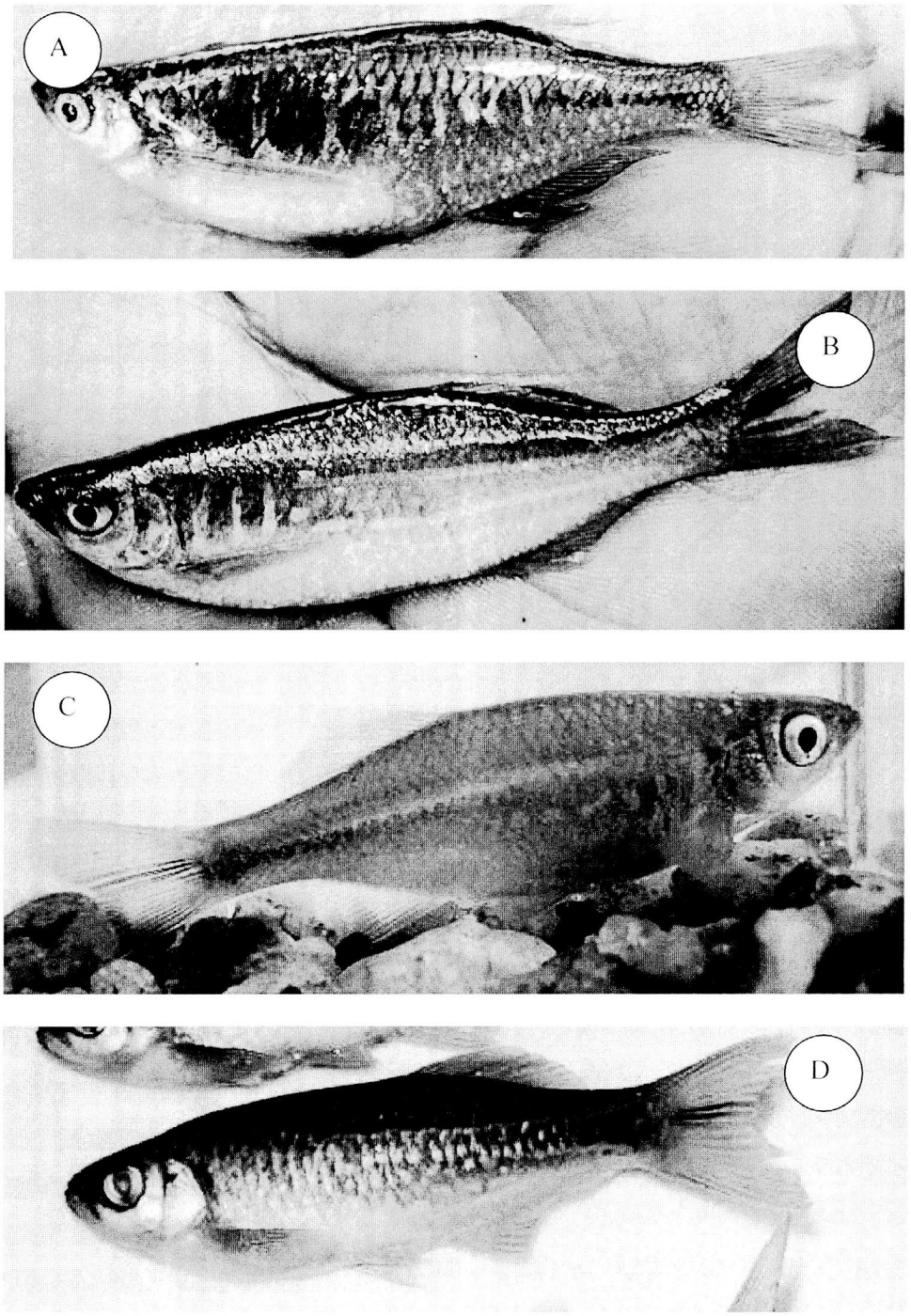


Figure 3-2. The genus *Devario* in Sri Lankan study streams; (A) *Devario pathirana* (Note characteristic "Y" shape vertical elements on the body); (B), (C) and (D) represent *Devario malabaricus* from Kalu-ganga (Rambukoluwa, N06°57'41.7" E80°56'09.7"), Uma-oya (Medawela, N06°56'33.9" E80°50'25.3") and Hatton-oya (Hatton, N06°54'10.8" E80°34'59.8") in the Mahaweli River basin, respectively (Note different vertical element patterns on body across locations. Phenotypically, the presence of "vertical elements" in the pigment pattern in deeper-bodied *D. malabaricus* is linked with adulthood (McClure and McCune 2003) (Photos by Jayakody A. Sumith).

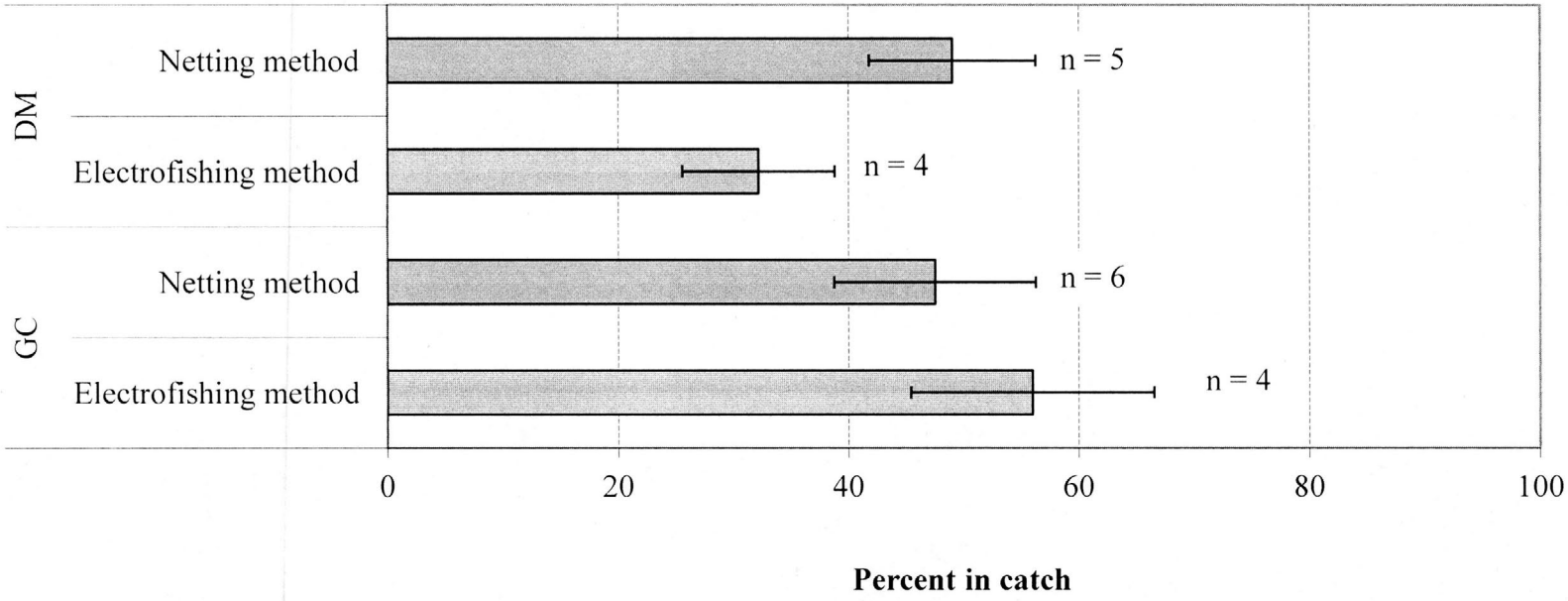


Figure 3-3. Total catch per reach length (= population) estimates (mean ± SE) by two sampling techniques, expressed as percentage of total (catchability) in stream sections in the Uma-oya. The number (n) of sampling trials in different stream sections are shown on the top of the bar. GC = *Garra ceylonensis* (represents benthic species); DM = *Devario malabaricus* (represents pelagic species).

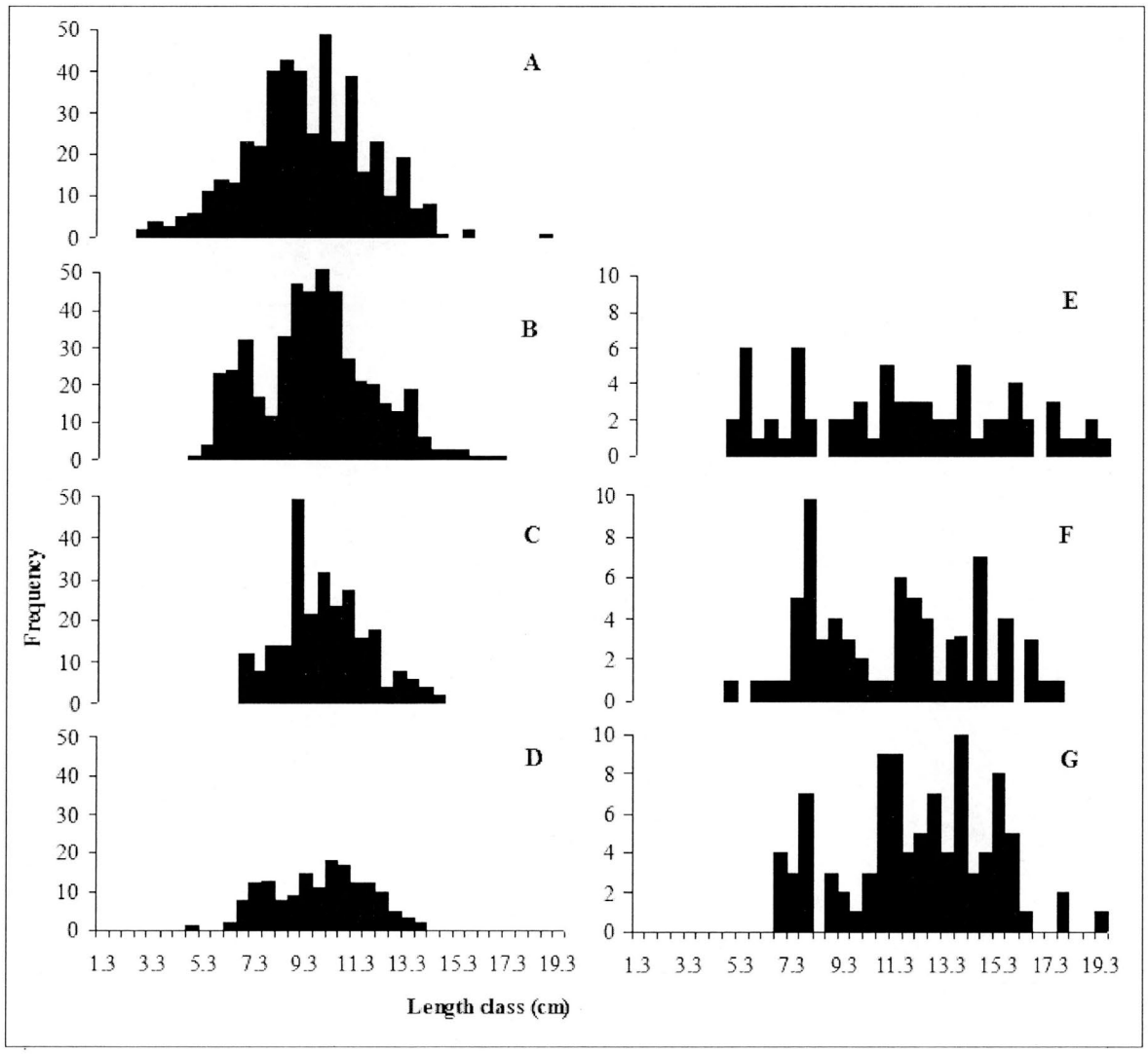


Figure 3-4. The length-frequency distributions of Ceylon stone sucker (*Garra ceylonensis*) sampled by electrofisher in February 2009 (A) and July 2009 (B) in Medawela and July 2009 (E) in Puhulpola. (C) and (D) sampled by using 10 mm mesh nets in September 2009 and January 2010 in Medawela, respectively. (F) and (G) are by 10 mm mesh nets in September 2009 and January 2010 in Puhulpola in the Uma-oya, upper Mahaweli River catchment, Sri Lanka. Total catch per sampling reach length (= population size), Length mean ± SD (A) N = 449, 9.5 ± 2.2; (B) N = 470, 9.1 ± 2.3; (C) N = 269, 9.8 ± 1.6; (D) N = 158, 9.8 ± 1.9; (E) N = 70, 11.4 ± 4.1; (F) N = 71, 10.9 ± 3.1; (G) N = 100, 12.2 ± 2.8. The skewness in probability plots of (A), (C) and (D) were unimodal, (B) was bimodal, and normally distributed ($p < 0.05$) while (E), (F) and (G) were staggered, and no distinct modality. Means of (C) and (D) are not significantly different by non-parametric Kruskal-Wallis test with *post hoc* Dunn's test ($p > 0.05$).

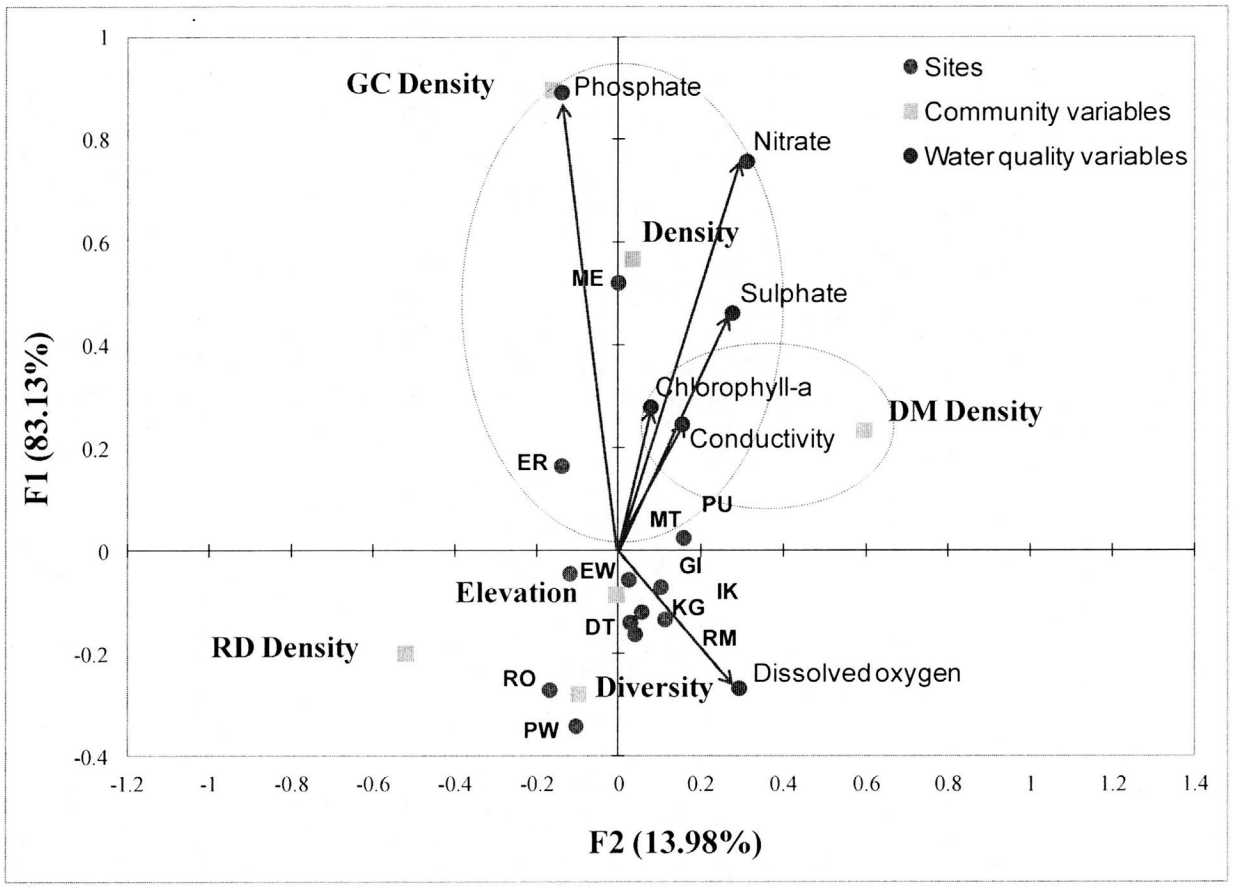
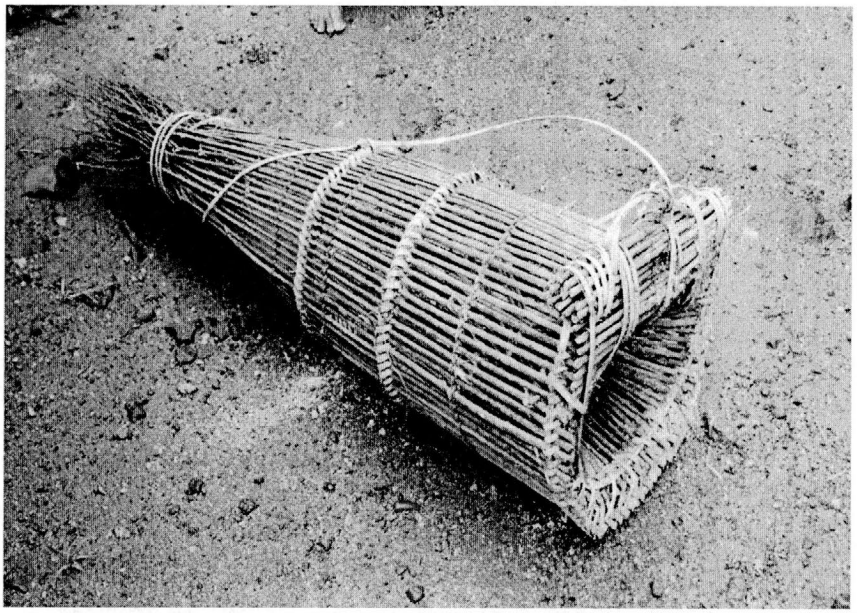


Figure 3-5. Plot of Canonical Correspondence Analysis (CCA) on stream water quality variables and total fish density and diversity parameters in selected sites in the Uma-oya and Knuckles streams. GC = *Garra ceylonensis* (represents benthic species); DM = *Devario malabaricus* (represents pelagic species); For descriptive site codes, see Figure 3-1 legend.



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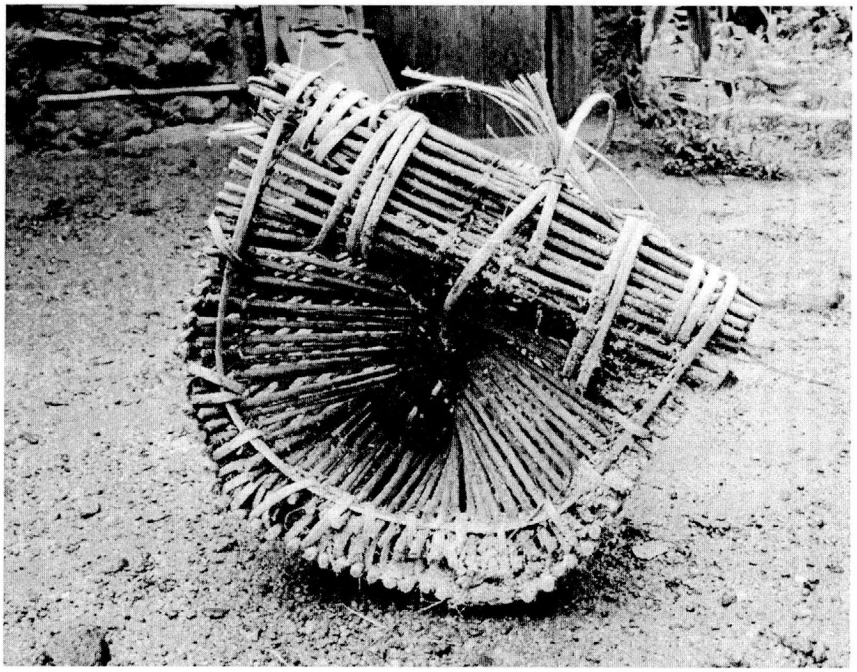

©Photo by Jayakody A. Sumith

Figure 3-6. Highly crafted fish trap "*kemana*" (above) and trap mouth (below), which is used to catch stream ascending *Garra* species during spawning migrations (Photos by Jayakody A. Sumith).

Table 3-1. Water quality (mean ± SD) and some habitat properties in sampling sites in the Uma-oya and Knuckles streams.[a]

Site[†]	Coordinates	Elev. (m) above MSL	Water quality						Habitat property			
			Condu. (µS cm^{-1})	DO (mg l^{-1})	NO^{3-} (mg l^{-1})	PO_4^{2-} (mg l^{-1})	Chlor. *a* (mg cm^{-2})	SO_4^{2-} (mg l^{-1})	Mesohabitat[♣]	Substrate[♠]	Aerial cover (%)[♥]	Riparian vegetation[♦]
Uma-oya (stream) [b]												
PW	N06°55'25.9" E080°51'49.4"	1,187	155	7.5 ±0.5	0.079 ±0.015	0.062 ±0.008	1.01 ±0.10	4.4 ±0.7	riffle>>pool>run	Boulders, rubbles, bedrock	50-80	90% giant cane, 10% bushes and tall trees
ME	N06°56'33.9" E080°50'25.3"	1,110	154	6.9 ±0.5	1.222 ±0.160	0.504 ±0.023	5.13 ±0.34	8.8 ±0.8	riffle>>pool>run	Boulders, rubbles, silt	5-10	70% giant cane, 30% bushes
ER	N06°53'00.3" E080°52'38.1"	1,106	173	6.6 ±0.3	0.553 ±0.103	0.259 ±0.029	1.42 ±0.13	5.4 ±0.5	pool>>riffle>run	Rubble, sand, silt	0-20	10% giant cane, 60% bushes and tall trees, 30% open space
DT	N06°51'24.7" E080°57'55.3"	1,058	114	7.2 ±0.5	0.317 ±0.021	0.123 ±0.014	4.29 ±0.30	2.6 ±0.2	pool>run>riffle	Boulders, silt, sand	30-60	100% bushes and tall trees
GI	N06°54'49.5" E080°53'12.5"	1,054	249	8.5 ±1.2	1.103 ±0.044	0.278 ±0.036	7.04 ±0.41	11.7 ±1.4	run>riffle>pool	Rubbles, sand, silt	40-60	100% giant cane
MT	N06°51'58.5" E080°57'11.8"	1,030	91	6.7 ±0.5	0.237 ±0.015	0.108 ±0.014	2.11 ±0.11	1.6 ±0.2	riffle>>pool>run	Boulders, rubbles, silt	5-10	40% giant cane, 10% bushes, 50% open space
PU	N06°54'45.9" E080°55'48.1"	981	236	7.1 ±0.5	0.886 ±0.147	0.094 ±0.019	1.02 ±0.13	9.5 ±1.3	run>pool>riffle	Boulders, sand, silt	10-20	90% giant cane, 10%

												tall trees
KG	N06°57'41.7" E080°56'09.7"	963	122	7.0 ±0.5	0.400 ±0.01	0.215 ±0.021	4.12 ±0.40	4.7 ±0.1	pool>>riffle>run	Boulders, silt, clay	30-50	40% giant cane, 60% bushes and tall trees
Knuckles streams [b]												
EW	N07°29'49.8" E080°44'57.8"	807	21	6.6 ±0.4	0.092 ±0.045	0.080 ±0.010	1.92 ±0.31	0.8 ±0.1	pool>>>riffle	Boulders, bedrock, sand, silt	20-40	100% bushes and tall trees (forest cover)
IK	N07°32'08.5" E080°46'19.0"	482	28	6.7 ±0.5	0.165 ±0.12	0.059 ±0.040	1.04 ±0.24	2.2 ±0.1	run>riffle>pool	Boulders, bedrock, rubbles	30-40	100% bushes and tall trees (forest cover)
RM	N07°30'11.8" E080°49'51.3"	339	31	6.8 ±0.5	0.061 ±0.08	0.091 ±0.041	1.17 ±0.22	1.4 ±0.2	pool>riffle>run	Boulders, bedrock, rubbles, silt	80-90	75% bushes and tall trees (forest cover), 25% grass
RO	N07°31'20.2" E080°47'57.0"	217	41	6.7 ±0.4	0.129 ±0.08	0.106 ±0.08	7.16 ±0.56	3.4 ±0.3	run>>riffle>pool	Rubbles, sand, silt	30-50	100% tall trees (forest cover)

[a] Each sample was replicated three times and averaged before the assessment. [b] Sampled in January, 2010

† For descriptive site codes, see Figure 3-1 legend.

♣ Mesohabitat classification was based on Didier and Kestemont (1996); the strength of coverage of each mesohabitat is denoted by (>)

♠ Substrate types were approximated to classification by silt (mud or clay, organic muck), sand, gravel (2–50 mm), rubble (50–200 mm), boulder (> 200 mm), or bedrock.

♥ Based on best personal judgment

♦ % contribution within 5 m from the stream margin

Table 3-2. Relative abundance of fish species present at preliminary sampling sites in the Knuckles and Uma-oya tributaries.[†]

Family/Species	English name (Sinhala name)	Relative abundance (% by number)											
		Knuckles streams				Uma-oya streams							
		IK	EW	RO	RM	PW	ME	GI	ER	PU	DT	MT	KG
Cyprinidae													
*Garra ceylonensis**	Ceylon stone sucker (Gal pandiya)	23.9	84.5	5.9	9.7	72.4	66.8	37.5	75.6	36.0	50.4	21.7	-
G. phillipsi[††]	Phillip's garra	?	?	?	?	?	?	?	?	?	?	?	-
Rasbora daniconius	Striped rasbora (Dandiya)	-	-	16.6	21.0	-	-	-	11.6	1.7	17.8	4.1	42.8
Devario malabaricus	Giant danio (Rath kailaya)	61.0	15.5	28.4	56.4	-	26.9	43.4	-	54.2	30.6	70.1	-
D. aequipinnatus[††]	Giant danio	?	?	?	?	-	?	?	-	?	?	?	-
*D. pathirana**	Barred danio	-	-	3.6	-	-	-	-	-	-	-	-	-
Tor khudree	Deccan mahseer (Leylla)	0.5	-	10.7	-	-	-	-	-	2.4	-	-	2.3
Puntius bimaculatus	Redside barb (Ipilli kadaya)	1.1	-	17.2	3.9	-	1.6	4.5	3.2	0.8	1.2	1.0	34.5
P. dorsalis	Long snouted barb (Katu kureya)	-	-	0.6	-	-	-	-	-	0.1	-	-	0.1
P. sarana	Olive barb (Mas pethiya)	-	-	-	-	-	-	-	-	-	-	0.1	-
*P. martenstyni**	Martenstyn's barb	13.5	-	10.1	-	-	-	-	-	-	-	-	-
*P. singhala**	Singhala barb	-	-	3.0	-	-	-	-	-	-	-	-	-
*P. kamalika**	Kami's barb	-	-	2.4	-	-	-	-	-	-	-	-	-
*P. srilankensis**	Blotched filamented barb (Dankudu pethiya)	0.3	-	1.2	-	-	-	-	-	-	-	-	-
Channidae													
Channa gachua	Snakehead (Paradal kanaya)	-	-	-	-	-	0.1	7.2	1.7	1.1	-	-	-
Channa orientalis	Walking snakehead	-	-	-	2.6	-	-	-	-	-	-	-	-

	(Kola kanaya)												
Clariidae													
*Clarias brachysoma**	Walking catfish (Magura)	-	-	-	0.4	-	-	-	-	-	-	-	-
Cobitiidae													
*Lepidocephalichthys jonklaasi**	Jonklaas's loach (Pulli ehirava)	-	-	-	0.8	-	-	-	-	-	-	-	-
Lepidocephalichthys thermalis	Common spiny loach (Ehirava)	-	-	-	-	27.6	3.7	4.7	0.2	2.6	-	-	-
Balitoridae													
*Neomacheilus notostigma**	Banded mountain loach (Puwakbadilla)	-	-	-	5.2	-	-	-	-	-	-	-	0.8
Osphronemidae													
*Belontia signata**	Ceylonese combtail (Thalkossa)	-	-	1.2	-	-	-	-	-	-	-	-	-
Cichlidae													
Oreochromis niloticus†	Nile tilapia (Theppeli)	-	-	-	-	-	-	-	-	-	-	-	19.5
Anguillidae													
Anguilla sp.	Eel (A'nda)	-	-	-	-	-	-	-	-	-	-	0.3	-
Poeciliidae													
Poecillia reticulata†	Guppy (Saree guppy)	-	-	-	-	-	0.9	1.7	7.7	1.1	-	0.9	-
Xiphophorus hellerii†	Green swordtail	-	-	-	-	-	-	1.0	-	-	-	1.9	-

For descriptive site codes, see Figure 3-1 legend.

‡Values are pooled average percent abundance of four sampling times in three sites (ME, GI, and ER), and PU where 3, RM where 2, and IK, EW, RO, PW, DT, MT and KG only one sampling estimates are given.

*Endemic fish species; †Exotic fish species; ††Reserved for possible sympatric existence in the Knuckles streams (Pethiyagoda 1991); ? = Unknown or data deficient.

Table 3-3. Fish diversity indices in sampling sites (for descriptive site codes, see Figure 3-1 legend).

Index	Uma-oya stream								Knuckles streams			
	PW	ME	ER	DT	GI	MT	PU	KG	EW	IK	RM	RO
No. of species (S)	2	6	6	4	7	8	9	6	2	6	8	11
No. of individuals/100 m^2 (mean)[†]	1.6	232.3	138.7	58.8	76.3	66.5	85.4	37.2	28.7	16.8	46.0	15.4
Shannon diversity (H')	0.59	0.93	0.73	1.07	1.17	0.90	1.07	1.13	0.43	1.01	1.20	2.01
Evenness (H'/lnS)	0.85	0.52	0.41	0.77	0.60	0.46	0.49	0.70	0.62	0.56	0.58	0.84
Dominance[♣]	-	0.93	-	0.81	0.81	0.93	0.91	-	1.0	0.85	0.70	0.34
Coefficient of Geographic Resemblance (CBR)								0.39				
Endemism (%)	50	16.6	16.6	25	14.3	14.3	11.1	20	50	25	50	61.5

[†]Data averaged to a single fishing effort where multiple sampling events performed.

[♣] Calculated for the most abundant pair of *Garra ceylonensis* and *Devario malabaricus*.

CHAPTER 4

4 Health status of Ceylon stone sucker (*Garra ceylonensis* Bleeker 1863; Teleostei, Cyprinidae) populations in an intensive agricultural catchment, Uma-oya, Sri Lanka[3, 4]

4.1 Abstract

Agricultural impacts on the associated aquatic ecosystem are primarily non-point and difficult to quantify. Growth, development, and reproductive success of fish populations respond to and signal important changes in the ecosystem. We examined population responses in Ceylon stone sucker, *Garra ceylonensis* populations to evaluate the impacts of multiple stressors in an agricultural catchment (Uma-oya, Sri Lanka). Samples were collected at varying elevations and compared at different locations within the catchment to differing levels of agricultural stress, and also to a reference catchment in a forest reserve. There was a strong eutrophication response in the agricultural catchment represented by increased size and higher reproductive investment in both male and female *G. ceylonensis* suggesting increased food availability. Sub-optimum gonad investment and fecundity of females at sites associated with high agricultural inputs may be due to metabolic disruption, and also may be influenced by high sediment influx. Future studies should focus on whether these changes are a consequence of an adaptive strategy to avoid sediment impacts, or simply reflects a seasonality effect, or it is a result of genetic diversity. Iterative studies will be required to examine specific responses of reproduction, and other indicators of reproductive health to agricultural exposures.

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4.2 Introduction

Intensive agriculture is associated with a variety of impacts on river systems including issues related to inputs of sediment, nutrients, chemicals, and changes in temperature (Vermeer et al. 1974; Cuffney et al. 2000; Wirth et al. 2001; Leight et al. 2005; Gray et al. 2005). Agricultural inputs to the aquatic ecosystem primarily originate in a diffuse manner, so the patterns and quantities of stress are difficult to predict (Munkittrick et al. 2000). There are various potential sources of stressors in agricultural catchments on fish, such as physical disturbance (suspended solids, temperature, pH), organic enrichment (domestic and urban discharges), toxic materials (inorganic and organic contaminants, heavy metals, pesticides), and development activities (channel modification, construction of dams and land development) (Scanlon et al. 2007; Ansara-Ross et al. 2009).

The Uma-oya catchment in Sri Lanka is characterized by extensive drainage systems associated with year-round agricultural practices. In monsoonal areas, surface and ground water chemistry have been shown to be affected by the rainfall. Modeling of NO_3^- pollution in the Uma-oya (stream) was strongly related to land use/cover and flow patterns (Dayawansa 2003). Ranasinghe et al. (2008) revealed excess aluminium (Al) and lead (Pb) concentrations in sediments close to agricultural and urban areas, and Bandara et al. (2011) reported mean concentrations of cadmium (Cd) in a range of 10.7 to 13.2 $\mu g\ l^{-1}$ in the Uma-oya, which he attributed to the use of triple-super phosphate fertilizers with high levels of Cd contamination.

Most studies of agricultural impacts in tropical areas have been laboratory-based and have focused on pesticide impacts using biomarker responses in wild fish (Wijeyaratne and Pathiratne 2006; Pathiratne et al. 2009). There have been reports of sub-lethal pesticide impacts on protein (Tripathi et al. 2003; Naveed et al. 2010; Al-Kahtani 2011), carbohydrate (Rani et al. 1989; Al-Kahtani 2011), and fat metabolism (Remia et al. 2008; Saradhamani and Selvarani 2009) in fish. Some groups of insecticides, such as organophosphates and N-methyl carbamates,

are known to inhibit acetylcholinesterase enzymes and neurotransmitter levels leading to fish behaviour impairments (Karen et al. 2001). These changes have been associated with defects in early life history forms (De Silva and Samayawardhena 2002; Colombo et al. 2005; Sumanadasa et al. 2008) and lethargy resulting in less preference for food and weaker escaping ability from predators (Bainy et al. 1996; Pan and Dutta 1998). Although, the impacts of environmental contaminants have been well documented in fish at the molecular, cellular, organ, and whole organism levels, few studies have focused on the higher levels of organization (Adams 1990, 2002; Siligato and Böhmer 2001, 2002). While the extrapolation of effects at the individual level to effects of population level is not an easy task (Adams 2002; van der Oost et al. 2003), stressor effect(s) at the population level need to be established in order to be ecologically significant (Gray 1989; Adams 1990, 2002; Adams and Greeley 2000; Crane et al. 2007).

Survival, growth, and morphometric responses in fish have been applied to studies of impacts of mining (Laflamme et al. 2000; Couture and Rajotte 2003), municipal and sewage discharge (Gibbons and Munkittrick 1994; Chambers et al. 2000; Galloway et al. 2003; Hewitt et al. 2005), industrial and pulp and paper mill effluent (Adams et al. 1992; Galloway et al. 2003; Sandström and Neuman 2003; Chiang et al. 2011), and agricultural pollution (Gray and Munkittrick 2005; Brasfield 2007; Smedley 2009). The survival, energy storage (e.g. condition factor and relative liver size), and energy expenditure (e.g. relative gonad size, egg size, etc.) are three key processes in any organism's life history (Munkittrick et al. 2000); these processes undoubtedly are interconnected.

We have recently developed a framework for examining fish populations in tropical areas using Sri Lanka as a case study of intensive agricultural practices and their influence on river systems (Chapter 2; Sumith and Munkittrick 2011a). Sri Lanka has 103 river basins draining from a central hilly mass (up to > 2,500 m above mean sea level (MSL). The Mahaweli is the longest river in Sri Lanka with 335 km-long and has a catchment area of 10,448 km^2 and

an annual discharge of about 8.4 billion cubic meters. The Mahaweli basin was subjected to an accelerated development project launched in the late 1970's, which brought about 127,000 ha of lands into year-round cultivation and another 182,000 ha aswaddumized (i.e. brought under cultivation) in two seasons (Tolisano et al. 1993). A post-project environmental evaluation report (Tolisano et al. 1993) showed system impairments from agriculture including (a) chemical inputs, which degrade local surface and ground water quality; (b) inappropriate cropping patterns for the soil and microclimatic conditions of the site, resulting in reduced productivity and increased reliance on environmentally inappropriate agricultural practices; and (c) poor water management practices, which have increased the threat of soil salinization and on-site soil losses. A survey of farming practices has shown that small-scale farming plots, and two to three cropping sequences per year resulted in the heaviest use of pesticides exceeding 23 kg ha^{-1} $year^{-1}$ active ingredients (Sumith and Munkittrick 2011b). Intensive human activities in the upper Uma-oya catchment have resulted in annual sediment yields (as measured by suspended river-load fluxes) of 130 to 2,100 t km^{-2}. The local rate of soil loss has been estimated as high as 7,000 t km^{-2} $year^{-1}$ in hill slope agricultural lands (Hewawasam et al. 2003), which exceeds the soil loss tolerance value of 5,000 t km^{-2} $year^{-1}$ (Gunathilake and Gopalakrishnan 1999).

The detrimental impacts of agricultural activity on aquatic biota have not yet been systematically assessed in Sri Lanka. Most field research and ecological studies in streams and rivers have looked for changes in diversity and abundance at the community level (e.g. Moyle and Senanayake 1984; Pet and Piet 1993; Weliange 2007), while none of these appear to be complemented by agriculture as a significant ecosystem modifier. Most laboratory studies have focussed largely on relatively short-term exposure to individual pesticides (e.g. Chandrasekare and Pathiratne 2005; Colombo et al. 2005), while in the majority of instances exotic fish were used as surrogates for indigenous species (Chandrasekare and Pathiratne 2005; Jayasundara and Pathiratne 2008). We recently demonstrated substantial changes in the fish communities in an

agricultural catchment, including increases in non-native species when compared with forested catchment in a nature reserve (Chapter 3; Sumith et al. 2011). Preliminary investigations evaluated a potential monitoring program using fish, and found only three species that were sufficiently widely distributed to be potentially used as sentinels for assessment of population level impacts. These species are *Garra ceylonensis*, *Devario malabaricus*, and *Rasbora daniconius* (Chapter 2; Sumith and Munkittrick 2011a). Previous studies on the genus *Garra* were limited to explicit descriptive biology of growth, development and reproduction (De Silva 1991; Yalçin-Özdilek and Güler-Ekmekçi 2006; Abedi et al. 2011). This study evaluated the fish state in prespawning *G. ceylonensis*, at a series of sites receiving seasonal stressors of agricultural inputs. This chapter will include unique performance parameters on growth and reproduction in *G. ceylonensis* in environments receiving agricultural effluents (runoff) and a reference catchment in a forested reserve.

4.3 Materials and Methods

4.3.1 Study area

The Uma-oya catchment of the upper Mahaweli river basin is said to be the area in Sri Lanka which is the most disturbed by human activities after the decades of land encroachment for tea cultivation and subsequent land use changes to vegetable cultivation in former tea lands (Chandrasekera et al. 2000). Agriculture covers most parts of the Badulla district and the stream is also influenced by three major townships of Nuwara-Eliya (7°0′0″N, 80°45′0″E), Welimada (6°54′4″N, 80°55′22″E) and Bandarawela (6°50′0″N, 80°59′0″E) with a total population of 874,000. The Uma-oya, with a catchment area of 740 km^2, is one of the main tributaries of Mahaweli River basin, which flows into Rantambe Reservoir, the last of the chain of reservoirs constructed under the Accelerated Mahaweli Development Project (AMDP) (1979–1984). The mean annual discharge is in excess of 14.5 (± 2.8 SD) million m^3 (as measured at the Welimada

gauging station during 1993 through 2004; Mahaweli Authority, Sri Lanka). The catchment receives 1,570 to 2,180 mm of rainfall annually (as measured in six stations during 2008 and 2010; Department of Meteorology, Sri Lanka). The air temperature maximum 28.7 and minimum 16.2°C were recorded during 2008 and 2009 in the catchment. In addition to agriculture, the catchment is affected by channelization for irrigation, fishery, tourism, hydro-electricity, and water supply for domestic and urban necessities.

The Knuckles reference catchment is characterized by montane forests in a buffer zone of approximately 260 km^2 area which spans from 200 to 1,900 m above MSL (Pinard et al. 2010). The Knuckles streams represent a part of the Amban-ganga catchment, which receives 1,435 to 2,111 mm of rainfall annually (as measured in six stations during 2008 and 2010). The maximum temperature of 31.5 and minimum of 20.2°C were recorded during 2008 and 2009 (Department of Meteorology, Sri Lanka). Intensive human activities in most of the areas have been stopped since declaration of the area as Conservation Forest in May 2000 by the Government of Sri Lanka.

4.3.2 Site selection and scoring criteria

Replicate sampling sites were selected from three catchment elevation levels in the Uma-oya (viz. upper, middle, and lower) representing an elevation gradient design (as proposed in Chapter 2; Sumith and Munkittrick 2011a), with minimum logistical barriers for fish sampling (i.e. easy access to the stream). The approximate land catchment area above each level was 48, 98 and 740 km^2, respectively (Hewawasam et al. 2003); the drainages were determined from a 1: 63,360 scale map (Survey Department, Sri Lanka). Fourteen sites were surveyed for fish community composition and 13 sites were identified with fair abundance of Ceylon stone sucker (*Garra ceylonensis*). All sites were considered as deteriorated in the Uma-oya, due to intensive exploitation for agriculture. A modified subjective scale of stress (Mayon et al. 2006) was considered in this assessment, where streams in agricultural tributaries were defined for five

criteria, (area under cultivation, hydrological regime, stream connectivity, stream mesohabitats (e.g. riffle sections are indispensable features in streams as preferred habitats of *G. ceylonensis*), and nutrient organic inputs), which were based on best expert judgement and/or based on existing datasets, and coded as 1 = high impact, 2 = medium impact and 3 = low impact. The sites PW (1-1), EP (2-1), HK (3-1), DT (4-1) and RP (6-1) were uppermost sites or lower-order tributary streams, which qualified for low impact category (3). The sites PU (1-4) and MT (2-4) were at confluences for one or more tributary streams and located in furthest downstream of Bomburuella-oya and Nanu-oya (two main tributaries of Uma-oya), respectively that qualified for high impact category (1). All other sites of ME (1-2), GI (1-3), GB (2-2), RT (2-3), ER (3-2) and MD (6-2) were considered medium impact (2) sites based on their position in between (1) and (3) categories.

Four altitudinal (elevation) categories were defined according to the following scheme: highest level as 1 = all sites > 1,150 m above MSL; 2 = all sites > 1,050 < 1,150 m; 3 = all sites > 950 < 1,050 m; and lowest level 4 = < 950 m above MSL. The global positioning system (Garmin GPSMAP® 76Cx portable GPS) was used to identify sampling stations along the main stream and tributaries and the values were accurate within ±3 m. Two sampling locations were considered as minimum in any of the scoring/elevation category levels. Two sampling sites from the Knuckles streams were considered as reference areas and they were from elevations of 482 m (Theligam-oya; Ilukkumbura N07°32'08.5" E080°46'19.0") and 217 m (Kalu-ganga; Rambukoluwa N07°31'20.2" E080°47'57.0") above MSL. Despite different elevation and reproductive seasonality of *Garra* spp., the reference sites were considered surrogates for population performance assessments since there were not any acceptable reference locations devoid of human impact within the Uma-oya catchment. Attempts to use sites along the Hatton-oya as reference sites for *Garra* spp. evaluated in 5 locations between Hatton (N06°54'10.8" E080°34'59.8", 1,170 m above MSL) and Rambukpitiya (N07°01'35.8" E080°30'35.4", 574 m

above MSL) on the stem stream of the Mahaweli River were not successful, as few individuals could be found. The distribution and relative abundance of *G. ceylonensis* in the study sites is presented in Figure 4-1.

4.3.3 Fish sampling and processing

Prespawning *G. ceylonensis* were sampled from 23 sites in three stream catchments over two years in January 2010 and 2011. In order to facilitate population and community-related assessments, quantitative samplings were conducted in all sites in pre-determined stream reaches employing three methods of netting (i.e. one cast net, two dip nets, and one drag net) and collecting all trapped fish. In order to standardize fishing efforts between sites, sampling methods were standardized as far as possible between sites by number of passes, time between consecutive passes, and efforts per unit area. Three upstream passes were conducted for each of three types of netting: (1) cast net was employed in consecutive and non-overlapping stream sections, approximately 4 m in length (= diameter of the cast net); (2) two dip nets were employed for stream sections in edges in a similar way approximately 50 cm in diameter; and (3) a drag net (1 m in width and 6 m in length) was employed for the middle stream sections. The stretched mesh size was 10 mm in all nets. Each section of sampling reach included a minimum of two riffles and mini pools. All sampling reaches were isolated at the top and bottom of each reach by stop-nets to prevent fish movement in or out of the area during sampling. When quantitative fish estimations for density parameters needed, methods described by Sumith and Munkittrick (2011a) were followed. The prespawning fish specimens from the Knuckles streams were collected in November, 2011 by passive traps (described in Chapter 3) during prespawning migration. The prespawning specimens were defined for the assessment, as the stage of which the ovary is filled with large, mature, fully-yoked oocytes (or milky-white testes) in final stages of maturation, just before the release of eggs or gametes (Tyler and Sumpter 1996).

All fish specimens were measured for total length (L) (± 0.1 cm) and total weight (W) (± 0.01 g) on site. Sample sizes were selected based on *a priori* power analysis in order to detect a 25% difference among gonadosomatic index (GSI) per site when $\alpha = 0.05$, $\beta = 0.20$ (Chapter 2; Sumith and Munkittrick 2011a). Only adult specimens of females > 8.5 cm and males > 7.0 cm of total length were sampled. All specimens were anaesthetized by eugenol and preserved in buffered formalin (10%) immediately after capture for organ weight (gonad and liver) analysis in the laboratory. A small slit on the anal opening was made before fixing in the formalin to enhance internal organ preservation. After measurements, all undersized specimens were released back to the stream below the sampling reach. In the laboratory, voucher specimens were measured for fork length (L) (± 0.1 cm) and total weight (W) (± 0.001 g) and dissected for gonad weight (± 0.001 g), and liver weight (± 0.001 g). All individual post-vitellogenic egg masses were preserved separately in containers with 70% alcohol for further processing. Five clumps of eggs were selected from either lobe of egg mass from three different regions of the ovary (i.e. cephalic, mid, and posterior) along the vertical direction, blotted-dried, and measured for weight (± 0.001 g) and counted per each fish. The absolute fecundity was calculated gravimetrically (Konswal 1990) by the following formula: F = [S] GW where; F = Absolute fecundity, [S] = Mean number of eggs/g of five samples, GW = Total gonad weight (g). Ten eggs from each clump were mounted on a calibrated glass slide and measured for diameter in micrometers (μm) under the low-power (10 x 10) microscope.

4.3.4 Water sampling and analysis

A maximum of fourteen sites in the Uma-oya (represented by agricultural catchment) and four sites in the Knuckles streams (represented by forested catchment) were sampled. Grab samples were taken from running waters of each stream section (reach) used for fish sampling. Seasonal differences of water quality were measured in April and July (months represented by low-flow months) and October and January (months represented by high-flow months).

Electrical conductivity, alkalinity and pH of surface water were analyzed *in situ*. Three pebbles (mean diameter: 16.5 cm) from three points along the transect of the sampling reach were sampled and transported in polyethylene bags on ice for chlorophyll-*a* analysis. Within 6 h following the sampling procedure, algal film was removed from the upper surface with a toothbrush and suspended in deionized-water. The scrubbed-surface of the pebbles was traced on tracing paper and its area (cm^2) calculated from the sum of the tracings. Chlorophyll-*a* measurement was performed using a mixed-suspension of algal films from the three pebbles, utilizing 90% acetone extraction on standard spectrophotometry (APHA 2005). In addition, water samples were taken in 500 ml pre-cleaned plastic containers, and transported on ice, and analyzed in the laboratory of Ecology and Environmental Biology, Institute of Fundamental Studies, Kandy, Sri Lanka for major water quality parameters using standard methods (APHA 2005). The hourly temperature profiles in selected locations of the study streams were measured by placing HOBO® water temp proV2 data loggers.

4.3.5 Sample treatment and statistical analysis

The relationships between length (L) and weight (W) were determined using best fit least square linear regression model performed on log-transformed data with W as dependant variable. Consequently, the length-weight relationships ($\log W = \log a + b \log L$) were determined for all mature individuals (a minimum length of mature male fish were 7.5 cm while female fish were 8.5 cm according to previous estimations; Sumith and Munkittrick 2011a). The allometric coefficient b (slope) of the length-weight relationship was tested for differences among sites with the theoretical value 3 using the analysis of *t*-test to assess whether these species exhibit isometric ($b = 3$) (cube law as proposed by Le Cren (1951) or allometric ($b \neq 3$) growth (Bobori et al. 2006). For each sex from each sampling location, physiological condition indices such as gonadosomatic index (GSI= 100*gonad weight/body weight), liversomatic index (LSI=100*liver weight/body weight) and Fulton's condition factor, K [100*(weight/length^3)]

were calculated. Mean egg weights were calculated from subsamples taken for fecundity estimations. Egg diameter represented a mean of 50 eggs (10/clump) from random sampling. Data are presented as mean±SE of mean or SD.

All measurements were log-transformed prior to comparisons among factors (sites, elevations, scaled sites, etc.) using ANOVA, with *post hoc* analysis of differences between factors using Tukey's HSD tests. Sexes were analysed separately. The Canadian Environmental Effects Monitoring (EEM) program-designated standard threshold levels, where any deviation in 25% of L, W, GSI, LSI and fecundity parameters or 10% deviation in K that exceed reference values were considered as ecologically relevant responses in fish (Walker et al. 2003). Statistical analyses were performed using Minitab® version 16 for Windows (Minitab, Inc.) and *alpha* levels were set at 0.05.

4.4 Results

The cyclical pattern of mass sedimentation rate of soil particles eroded from the Uma-oya catchment measured at the gauging station at Welimada (6°54'96"N 80°55'22"E) is shown in Figure 4-2. Because of the pesticide application patterns and intensity of agriculture (Sumith and Munkittrick 2011b) all sites are considered to be contaminated in the Uma-oya. There were no drastic changes in the temperature profiles in the study streams, where Perawella (1,187 m above MSL) site in the Uma-oya recorded annual mean of 21.0°C (± 0.3 SD) with the lowest temperature observed in January. The minimum temperature was gradually increased up to May (20.2°C) and maximum temperature was recorded in June (24.1°C). Mean temperature recorded at Welimada (1,078 m above MSL) in the Uma-oya was 21.4°C (± 1.1 SD), while Etanwala (807 m above MSL) in the Knuckles stream was 20.5°C (± 0.8 SD). The Knuckles sites were assumed to be the least contaminated and best reference sites since the rainfall and temperature profiles were similar (Figure 4-3a–c). The reproductive pattern of *Garra* sp. was different with spawning

occurring in November rather than in January in the Uma-oya (see Chapter 2). However, the characteristics of the samples at spawning time were considered comparable for *Garra* in the absence of further information.

4.4.1 Agricultural influence on water chemistry

Attempts were taken to understand the seasonal changes[5] by sampling in months representative on northeast (NE) monsoon (October and January) and southwest (SW) monsoon (April and July) in both catchments (Table 4-1). The water conductivity was highest in the SW monsoon months reaching 158 to 193 $\mu S\ cm^{-1}$ in the Uma-oya, but in the same system, the values in the NE monsoon months remained in the range of 112 to 119 $\mu S\ cm^{-1}$. However, the conductivity in the Knuckles streams was considerably lower at a range of 30 to 33 $\mu S\ cm^{-1}$. A more or less similar trend was observed with respect to other parameters such as total alkalinity and total organic carbon in the Uma-oya; the SW monsoon range from 91 to 114 mg l^{-1} and 9.93 to 10.60 mg l^{-1}, respectively; the NE monsoon range from 25 to 53 mg l^{-1} and 4.34 to 6.97 mg l^{-1}, respectively. But, in the Knuckles streams, alkalinity was more or less similar in both seasons, and narrowly ranged at 19 to 21 mg l^{-1}. Total organic carbon was at a range of 1.04 to 1.76 mg l^{-1} during the same months. The average concentrations of nitrate-N in the Uma-oya streams during SW monsoonal months were in the range of 0.77 to 86 mg l^{-1}, while NE months showed higher concentrations in a range from 1.12 to 1.91 mg l^{-1}. In contrast, total phosphate concentrations in the SW months were higher than NE months in the range of 0.22 to 0.24 and 0.12 to 0.18 mg l^{-1}, respectively. Total phosphorus in the Knuckles streams across seasons varied from 0.08 to 0.09 mg l^{-1}. Several other parameters were also elevated in agricultural catchment such as total

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[5] Sri Lanka experiences two major agricultural seasons due to the monsoon rains. The *Yala* season receives rainfall from the southwest monsoon during April–July, while the *Maha* season receives rainfall from the northeast monsoon in October–January.

nitrogen and chemical oxygen demand (Table 4-1), when compared to forested Knuckles streams.

4.4.2 Chlorophyll-a

The concentration of chlorophyll-*a* assessed on stream substrates (e.g. cobble-size stones of 15 to 20 cm in diameter) were higher in SW-monsoon months in July (2009; 3.12 mg cm^{-2}) and increased up to 3.94 mg cm^{-2} in April (2010) in the Uma-oya streams. Of NE-monsoon months, October (2010) gave the lowest concentration at 0.99, whereas in January (2010) it was higher at 1.53 mg cm^{-2}. The estimates at the Knuckles streams during NE-monsoon month of January (2010) was 1.33 mg cm^{-2}, which was comparable with the concentration of the Uma-oya. The highest value of 5.31 mg cm^{-2} was from the Knuckles streams in April (2010), which has been the highest in two catchments (Table 4-1).

4.4.3 Seasonal fish sampling

4.4.3.1 Female *Garra*

Due to the nature of the study, optimum gonadal growth for agricultural impact study was determined by seasonal sampling in February, July, September (2009), and January (2010, 2011). Additional fish samplings were conducted in March (2009) and November (2010) in the Knuckles streams (Figure 4-4). Females (n = 24) in the Uma-oya had gonad investment of 7.1% (± 0.91) in February (2009) (total length and total weight (mean ± SE) were 12.3 ± 0.5 cm and 24.0 ± 2.9 g, respectively. A GSI of >1% was used to effectively discriminate mature individuals from immature fish, which has been used as guidance for separating fish for analysis in the Canadian EEM program (Environment Canada 2010). Gonad investment in January (2010 and 2011) over September (2010) was highly significant ($p < 0.001$), and increased by 12-fold (Table 2). Alternatively, a significant recession ($p < 0.001$) of gonad investment by almost one-half was observed in February over January.

Prespawning female size (length and weight) in July and September were significantly higher ($p < 0.001$) than January (2010 and 2011) sampling in the Uma-oya. In addition to these differences, female condition (K) was also significantly increased ($p < 0.001$) by over 6 to 8% and LSI by 19 to 22% between July and January (Table 4-2). The post-spawning GSI (< 2%) of *G. ceylonensis* in September in the Uma-oya suggests that gonadal investment must start during or after the month of October.

4.4.3.2 Male *Garra*

In males, no significant differences ($p > 0.05$) were observed in length between September (2009) and January (2010) or weight between July, September (2009) and January (2010) in the Uma-oya. However, male fish length in July was significantly lower ($p < 0.001$) than all fish in January sampling. Male fish in January (2011) sampling were the lowest in length and weight in all months (Table 4-2).

In January (2010, 2011), male fish in the Uma-oya had highly significant ($p < 0.001$) prespawning gonad development over post-spawning GSI in September fish by a factor of >8 (Table 4-2). In February (2009), males (n = 18) had gonad investment of (mean ± SE) 7.4% ± 0.78 at a total length of 9.1 ± 0.3 cm and a weight of 8.6 ± 0.8 g. An elevated GSI of >6 times was observed in February compared to post-spawning GSI in September. However, maximum GSI of 10.4% (± 0.42 SE) recorded in January (2010) was not significantly different ($p > 0.001$) than that of 7.4% (± 0.78 SE) in February (2009). A significant increase of condition (K) by 6.5 to 9% between September and January (2010 and 2011) was observed. Similarly, LSI was also increased by 21 to 41% between July and January ($p < 0.001$). However, larger livers in male fish were persistent during prespawning months (January) and during the spawning (February), as was observed for GSI (Table 4-2).

Length-frequency data of *G. ceylonensis* populations at sites of Girambe, Erabadda and Medawela in the Uma-oya showed that young-of-the-year (YoY) below 9.8 cm length class had

more than 62% individuals. With the year progression, there was a progressive decline of YoY of *G. ceylonensis* in Girambe, Erabadda and Medawela up to 14.9, 41.2, and 53.1%, respectively. However, this progressive decline of YoY in Medawela site was considerably low where YoY size class (<10.3 cm) had considerable representation by 71.9% in July and 56.9% in September of the total population. Obviously, least rejuvenating trend has been shown in Girambe (Table 4-3).

4.4.4 Prespawning sampling

January sampling was chosen as the maximal gonadal development in the Uma-oya, and a total of 403 of prespawning female *G. ceylonensis* were collected in January (2010 and 2011) from 12 locations in the Uma-oya mainstream and tributary streams; per site sample size (n) was in a range of 13 (minimum when one season sampling was accounted) to 51 (maximum when two sampling seasons were combined), consistent with the *a priori* determined sample size of 10 to 19 at 80% power ($\beta = 0.20$, $\alpha = 0.05$) (Chapter 2; Sumith and Munkittrick 2011a).

There were no significant differences in the K, LSI and GSI between prespawning *G. ceylonensis* in both sexes in January (2010 and 2011 in ME, GI and PU sites in the Uma-oya (female: pooled GSI, $p = 0.151$; LSI, $p = 0.984$, K, $p = 0.590$ and male: pooled GSI, $p = 0.097$, LSI, $p = 0.175$, K, $p = 0.880$). Thus, the measurement data of January (2010 and 2011) were combined for analysis of variance (ANOVA) in both sexes.

4.4.4.1 Female *Garra*

Although, Knuckles prespawning occurred in November (and in January in the Uma-oya), the condition (K), GSI and LSI data suggest as baseline in reference systems. There were significant differences (ANOVA, $p < 0.05$) of female fish size (in terms of mean length and weight) between the Knuckles and the Uma-oya. Female *G. ceylonensis* collected from RP (6-1) were heavier ($p < 0.001$) by 42% and PU (1-4) by 62% than the Knuckles (mean of two sites).

Females from ME (1-2) were 34% lower in condition ($p < 0.001$) than the Knuckles females. However, PW (1-1), GB (2-1), HK (3-1), ER (3-2) and DT (4-1) sites showed significantly smaller body mass by 14 to 38% compared with reference Knuckles females ($p < 0.05$) without length being compromised. In contrast, female length decreased significantly ($p < 0.001$) by 13% in ME ($p < 0.001$) and increased ($p < 0.001$) by 14 to 18% in RP and PU. The condition for prespawning at all sites was about 1.33% (± 0.02 SE), with an average GSI between sites of 17.9% (± 0.5 SE) and LSI of 0.64% (± 0.02 SE) (n = 53 females).

Comparing across all sites in the Uma-oya, there was no significant difference between years in condition or gonad size, but sites in some tributaries showed larger liver sizes in January 2010 or 2011. Since these differences represented only further increases in size, the data for these sites (MT, 2-4 ER, 3-2 and DT, 4-1) were also pooled and under-represent the increases in liver size in some years. On the main-stream of the Uma-oya (PW, 1-1 to PU, 1-4), fish size increased at each site downstream with the fish at the mouth of tributary-1 being larger than all other sites on that branch. When all elevations above 700 m were examined, there was a highly significant negative correlation between female fish weight and elevation ($r^2 = 70.1\%$, ANOVA, $p < 0.001$, $df = 10$). All sites in the Uma-oya showed prespawning liver sizes at least 30% larger than in the Knuckles region when fish were prespawning, and within the agricultural region two of the headwater sites (PW, 1-1 and HK, 3-1) had liver sizes more than double (i.e. > 114%) the weight of the Knuckles fish. Prespawning energy reserves in liver (LSI) were low as less than 1% in ME (1-2), GB (2-1), and MD (6-2) in the Uma-oya and were significantly different ($p < 0.05$) from rest of the sites in the catchment. No sites in the Uma-oya drainage had prespawning gonad sizes as large as the Knuckles *Garra* sp. and five sites had ovaries more than 25% smaller, and three of these were headwater sites (sites PW (1-1), GB (2-1), DT (4-1), MT (2-4) and MD (6-2) (Figure 4-4).

Contamination and elevation categories showed significant differences ($p < 0.001$) in fish size (L and W), gonad size (GSI), and liver size (LSI) in females. Interestingly, two categories appear to be responsive in opposite directions, where significantly heavier and bigger fish ($p < 0.001$) were associated with high contamination category, while medium and low categories showed no significant size differences from each other (Table 4-4). There was a significant size increase (in terms of length and weight of female fish) below 1,050 m elevation than above two elevation categories (Table 4-4). The lowest gonad size (GSI) showed in high contamination category while GSI of the elevation category 3 (> 950 < 1,050 m above MSL) was significantly ($p < 0.001$) lower than two higher elevation categories. The lowest liver size was observed in medium contamination category, which was 11% lower and 15% higher than high and low contamination categories, respectively. The liver sizes were augmented with increasing elevations and the size difference between the lowest elevation and highest elevation category was 44%. There were no significant differences ($p > 0.05$) of fish condition (K) whatsoever among any of the categories.

Female (n = 53) fecundity parameters of reference data were estimated; the number of eggs per female was 2,357 (± 115 SE), mean egg weight was 1.89 µg (± 0.04 SE) and mean egg diameter was 1.56 mm (± 0.02 SE) (Figure 4-5). The larger and heavier females had significantly higher fecundity (i.e. number of eggs per female fish) of high contamination category in the Uma-oya. However, higher fecundity at high contamination category at 2,614 (±140 SE) and the fecundity at the low contamination category at 2,245 (±97 SE) were not significantly different (Table 4-5). Similarly, bigger and heavier females below 1,050 m had significantly ($p < 0.001$) higher fecundity than higher elevation females at >1,150 m above MSL. Alternatively, the females at elevations > 950 and < 1,050 had significantly lower GSI value than higher elevation individuals. The regression of GSI and body weight of prespawning females of *G. ceylonensis* was highly significant among elevation categories (ANOVA, $p <$

0.001, n = 612). Yet, there was a significant negative trend of fish weight (W) and GSI (Pearson's correlation -0.143, $p < 0.001$).

4.4.4.2 Male *Garra*

A total of 232 prespawning male fish were collected in January 2010 and 2011 and sample size (n) ranged from 7 to 52 per site. Male *G. ceylonensis* was slightly to strongly less abundant compared to females in some locations; HK (3-1), RP (6-1) and MD (6-2), where they were precipitously less, while ER (3-2), DT (4-1) and GI (1-3) were slightly less (data not shown). Therefore, the sample size of males at less-abundant sites were slightly less than the minimum sample size of eleven, even when pooled at sites for two prespawning seasons (Chapter 2). The baseline data for reference condition (K) in respect to male *G. ceylonensis* was 1.20 (± 0.2 SE), while GSI of 6.72% (± 0.43 SE), and LSI of 0.71% (± 0.03 SE), were estimated (n = 26 males). All male fish in the Uma-oya locations except PU (1-4) site were smaller (12–33%), and skinnier (18–68%) than the reference Knuckles fish (ANOVA, $p < 0.001$). However, males collected from RT (2-3) and GI (1-3) had lower body mass without showing a significant length difference than the reference Knuckles fish. Males were smallest in the uppermost site (PW, 1-1) in the Uma-oya, and male fish in PU (1-4) had comparable body size with the Knuckles males (Table 4-5).

Male fish in all sites in the Uma-oya had liver weights (LSI) which were larger by 17 to 111% compared with the Knuckles males (Figure 4-7); Medawela (1-2) site had livers heavier than the Knuckles male fish, but only less than 25% (Figure 4-6). Two sites (ME, 1-2 and GB, 2-1) had prespawning liver sizes (LSI) less than 1%. They were significantly different ($p < 0.05$) from a headwater location at PW (1-1), and a confluence location at PU (1-4) and not significantly different ($p > 0.05$) from the Knuckles male fish. Liver was 1.5 to 2.1 times larger in all sites except ME (1-2) and MD (6-2) than Knuckles males. No condition (K) in any of the sites in the Uma-oya had significant differences ($p > 0.05$), but some sites had higher conditions

of GB (2-1) at 11.7%, MT (2-4) at 17.5% and ER (3-2) at 14.2%, compared with the Knuckles (Table 4-5).

The largest and heavier male fish were observed in the high "contamination" category ($p < 0.001$) and there was a gradient response across fish weight in both contamination and elevation gradients (Table 4-5). There was a progressive increase in fish size down the elevation categories 1 through 3. The fish above 1,150 m elevation showed the smallest size male fish ($p < 0.001$) compared with the lower elevations. Neither contamination scale nor elevation gradient were associated with differences in gonad size and condition in males ($p > 0.05$). The liver size in medium contamination sites was depressed by 22 and 25% compared to high and low contamination scales, respectively. The highest liver size was observed in the highest elevation category, which was 49% augmentation compared with the lowest elevation category (< 950 m above MSL). There were significant ($p < 0.001$) liver augmentations by 39% between elevation category 1 over 2 and 18% between 3 over 2 (Table 4-5).

The male GSI in all sites except the lowermost site (MD, 6-2) had larger gonads compared to Knuckles reference population. There were no significant differences found among sites in all elevations in gonad size. The uppermost site (PW, 1-1) in the Uma-oya had smallest fish in terms of length and weight, but had the biggest liver size, more than twice than Knuckles (Table 4-4). Another headwater stream tributary (HK, 3-1) in the Uma-oya also had nearly doubled (>1.8 times) liver size than Knuckles. This pattern is similar to headwater females in the Uma-oya. For male fish weight was also significantly negatively correlated for all elevations ($r^2 = 76.0\%$, ANOVA, $p = 0.002$, $df = 8$).

4.4.5 Alternative predictors of health

Headwater areas showed much lower density of *G. ceylonensis* than sites further downstream; on the Uma-oya mainstream (Bomburuella-oya) sites, standardized collection efforts showed density at the PW (site 1-1) at 114-times less of ME (site 1-2) further

downstream. On the main tributary (Nanu-oya), *G. ceylonensis* density was 32-times less at EP (site 2-1) at upstream than MT (2-4) at downstream. Similarly, a 12-times density drop was observed between upstream HK (site 3-1) and downstream ER (site 3-2) on a sub-tributary (Figure 4-1).

Linear regression of log-transformed data on W and L of *G. ceylonensis* were highly significant ($p < 0.0001$) for all thirteen sites in the Uma-oya. Regression slopes (b) were significantly different from isometry ($p = 0.001$) at sites EP (2-1), HK (3-1), RT (2-3) and RP (6-1) and the range of b was 2.93 to 3.10 with r^2 in a range of 0.965 to 0.988 (Table 4-6). The rest of the nine sites had lower b values (2.64–2.83) with r^2 between 0.903 and 0.984. Interestingly, three out of four sites, which showed isometry were headwater streams in the Uma-oya and were tributary streams. Prespawning (November, 2010) condition of *G. ceylonensis* ($n = 79$) was shown to be isometric with a b value of 3.03 ($p = 0.001$) and r^2 of 0.934 ($p < 0.0001$).

Mature individuals ($\geq$ 8.5 cm for females; $\geq$ 7.0 cm for males) of *G. ceylonensis* were sexed in January (2010) since gonad growth of both sexes was conspicuous at this time. Female dominance was observed in January (2010) in almost all sites except PU (1-4); the values at other sites varied from 0.05 to 0.27 as a proportion (Table 4-7). Female bias was significantly stronger ($p < 0.01$) than the reported mean sex ratio of 0.54 and the hypothetical ratio of 1.0 ($p < 0.0001$), except at PU (1-4) and MT (2-4) (Uma-oya stream locations), Rambukoluwa (Knuckles stream), and only PU (1-4) was not significantly different from the ratio suggested of 0.54 ($p = 0.427$).

The size of the mature male *G. ceylonensis* was not significantly different across the Uma-oya and Knuckles sites (Table 4-7). Total lengths of female *G. ceylonensis* in GI (1-3) and PU (1-4) in the Uma-oya were significantly ($p < 0.0001$) longer than that of the Knuckles counterparts; the longest female *G. ceylonensis* was 19.9 cm from the PU site (mean length 14.6 cm, $n = 25$) and the longest from the Knuckles streams was 15.4 cm. Correspondingly, the

heaviest female found was 70.4 g in total weight (mean ± SD 33.6 g ± 13.7, n = 25) in the PU site. The mean weight in the Knuckles females was at 14.4 g (± 6.7, n = 47) (data not shown). Mean rank density of *G. ceylonensis* in the Uma-oya stream was significantly higher (H =15.84, p = 0.003, df = 5) in several locations in a range of 27 to 144 (individuals/100 m^2) in contrast to the Knuckles streams (8 individuals/100 m^2; range 1 to 24). In terms of biomass production per unit area, *G. ceylonensis* alone represented over 16 g/m^2 in two stream locations in the Uma-oya (Table 4-7). Fish density estimates did not show linear relationships with environmental variables (e.g. nitrate, phosphate, sulphate, chlorophyll-*a* content) (p = 0.189) (Chapter 3; Sumith et al. 2011). However, there were significant associations of nitrate, phosphate and chlorophyll-*a* content with the overall fish density and *G. ceylonensis* density in ME (1-2), PU (1-4) and ER (3-2) sites ($p < 0.05$).

4.5 Discussion

Intensive agriculture has affected stream chemistry in the Uma-oya catchment over the years (Dayawansa 2003; Bandara et al. 2011). Erosion of soil by heavy rainfall events is by far the most influential means of entry of land-based agricultural contaminants into aquatic ecosystems. However, total suspended solid concentrations in the Uma-oya may not well represent the serial events of sediment loading as the samplings were done in low-flow conditions as grab samples. The primary physical and chemical indicators of agricultural effects are nutrients, pesticides, conductivity, and substrate quality (embededness), all of which tended to increase with agricultural intensity in this study. The Uma-oya showed elevated levels of conductivity, total alkalinity, total organic carbon, nitrogen and phosphate compared with the reference catchment in the Knuckles. The external inputs in stream water were much more pronounced in the southwest (SW) monsoon (*Yala* season) than northeast (NE) monsoon (*Maha* season) suggesting contaminant dilution during the higher rainfall periods. Despite the

possibility of dilution during NE monsoon months, high concentrations of nitrate-N in stream water indicate excess nitrogen loading and nutrient enhancement effect in the system.

4.5.1 Food availability and impact on G. ceylonensis

The seasonal chlorophyll-*a* concentrations on substrates across catchments suggest that the stream productivity may be limited by factors other than nutrients. During the NE monsoon months of October to January, the stream discharge and sediment loading is at its peak in the Uma-oya. The months of April (pre-monsoonal) and July (post-monsoonal) are relatively low-flow months and stream transparency would be minimally impacted, which could result in higher epilithic algal growth and chlorophyll-*a* levels. The inverse relationship between the transparency and the concentration of chlorophyll-*a* has been shown by Jones and Knowlton (1993).

There is a positive association between epilithic chlorophyll-*a* content and nutrients such as nitrogen and phosphorus (van Nieuwenhuyse and Jones 1996; Schindler et al. 2008; Conley et al. 2009). Female *G. ceylonensis* in the Uma-oya are longer and heavier than the reference sites, which suggest ample availability of preferred food in the agricultural streams. Epilithic chlorophyll-*a* concentrations estimated per unit substrate area showed a significant relationship with the total fecundity of *G. ceylonensis*. Dejenie et al. (2009) and their earlier work have shown a positive association between *Garra* biomass and total phosphorus in reservoirs. An increase in the food availability as would be expected in a eutrophic environment generally results in an increased growth rate, reproductive output, and density (Gibbons and Munkittrick 1994). Though this estimate of increased food availability is lower than the estimation given by De Silva (1991) in a low elevation stream in the Mahaweli River basin, the impact of increased food resources at the higher elevations in this study in the Uma-oya would be sizeable. This study showed that fish were larger at lower elevations so potential effects of eutrophication at the lower elevations would be more difficult to detect. The study by De Silva (1991) and several

other studies (Vannote et al. 1980; Edds 1993) have shown progressive increase in biomass downstream, thus a relatively smaller ichthyomass could be expected in small streams at higher elevations (Edds 1993).

Female *G. ceylonensis* tend to be vulnerable to gillnet fisheries due to the increased size of females in general (De Silva 1991) and such size-selective harvesting can result in sex-biased populations (e.g. Oliveira et al. 2000). There are other possible causes for altered sex ratios including differential mortality assuming that males have a shorter lifespan than females (De Silva 1991) or female-biased sampling. The selected maturity size of *G. ceylonensis* in the present study was in consensus with previous estimates of size-at-maturity under captive breeding as 7.5 to 8.5 cm (Sundarabarathy et al. 2005) and 9 cm under natural conditions (De Silva 1991).

Several indicators of a response to increased productivity such as increases in fish density, body size, liver and gonad size in agriculture-influenced sites have further supported the conclusion of an overall nutrient enhancement effect. Increased productivity in receiving water ecosystems in agricultural catchments has been shown that in areas with enrichment of nutrients and organic matter (Gray et al. 2002; Ansara-Ross et al. 2009). Agricultural and mining inputs can be enhanced during high flow periods. In contrast, impacts of point sources such as industrial (e.g. pulp and paper mill effluents) and municipal wastes occur during low-flow periods (Chiang et al. 2011).

Garra ceylonensis shows active gonadal development stages in November-December (*Maha* season) coinciding with peak rainfall events. In tropical climates, the seasonality of agricultural impact is a reality in most catchments because seeding and/or planting coincides with the rainfall pattern and increases with pesticide use. Such inputs also coincide with key developmental periods for fishes. With the advent of heavy rainfall events, streams in agricultural landscapes are subjected to a complex array of simultaneous stressors associated

with increased runoff that results in increased sediment, nutrient, chemical and pesticide loading (Brasfield 2007; Ansara-Ross et al. 2009). In addition, following heavy rainfall events in the Uma-oya catchment, irrigable lowland fields are prepared for rice cultivation during December-January. During successive land preparation activities, a copious amount of water is used for primary and secondary tillage, puddling and land leveling, and then allowed to drain into adjacent streams at the end of each event. Such activity can be viewed as an episodic event related to agricultural stressors when bound pollutants can be re-suspended and transported with the tail-water. In addition, young-of-the-year recruitment occurs during late February through April resulting in additional stress on young *Garra* populations. If, *G. ceylonensis* spawn in response to dry periods, as has been shown by De Silva (1991), theoretically maximum YoY individuals must be present in either February through April or July through September (during two dry spells after each monsoonal periods). If we allocate a one-year lead time from spawning to first maturity (Sundarabarathy et al. 2005), on yearly dimensions, the above time period would not be drastically affected. Therefore, it can be deduced from the present results that the main spawning season of *G. ceylonensis* in the Uma-oya coincides with the dry spells lasting from February through April. This finding is corroborated with the data of De Silva (1991). Development of key sensitive periods of gonadal growth and recruitment of early larval stages during active growing seasons are important to assure fish were exposed to agricultural effluents or stressors of interest. Brasfield (2007) noted that fish species such as slimy sculpin (*Cottus cognatus*) inhabiting agricultural streams are less suitable for evaluating impacts despite its abundance and site fidelity (Gray et al. 2002; Gray and Munkittrick 2005) because of peak exposures to stress which occur during periods of reproductive inactivity.

4.5.2 Impact on reproduction and timing

Maximum gonad development of female *G. ceylonensis* in the Uma-oya catchment occurs in January. Sumith and Munkittrick (2011a) reported lowest variability (CV 19–26%) and

strongest gonad weight-body weight relationship (r^2 = 0.54–0.67, $p < 0.001$) in females in January (2010) in the Uma-oya (Chapter 2). The seasonal peak in gonad development in *G. ceylonensis* populations has been documented in an alternate catchment in the Mahaweli River basin in Sri Lanka (De Silva 1991). This observation provides strong evidence that there is a seasonal maximum in gonadal activity during January as was found in this study and has been shown previously for *G. ceylonensis* (De Silva 1991; Sundarabarathy et al. 2005; Sumith and Munkittrick 2011a) and *G. rufa* (Patimar et al. 2010).

Spawning of reference *Garra* populations in the Knuckles streams occurred in November, and the lowest GSI levels were observed in January, despite similar water temperatures and rainfall intensities between catchments. Differences in reproductive timing between the two catchments required that the stages of reproduction be compared at a similar stage of development (i.e. fish at prespawning stage), rather than at the same time of year. Teleost fish species are remarkably successful in coordinating the timing of optimal reproductive investment in response to environmental signals (Helfman et al. 1997; Bass and Grober 2001) even though genetic makeup plays a fundamental role in gonad development (Helfman et al. 1997). In Sri Lanka, a major rainfall season (*Maha*) impending in October through December is a strategic season for riverine fishes for spawning. The differences between the catchments suggest that these populations may represent a separate subspecies between the catchments, or the differences in reproductive timing could be an adaptive strategy to avert sediment deposition periods in the Uma-oya. The lack of unexposed Uma-oya populations places limitations on the interpretation of the basis for the differences.

In prespawning males, despite a strong relationship of gonad weight-body weight (r^2 = 0.66–0.87, $p < 0.05$ to < 0.001) in multiple locations in the Uma-oya (Sumith and Munkittrick 2011a), the variability was relatively higher (coefficient of variation 20–30%) in January implying individual variability in spermiation. Although female GSIs were markedly reduced in

February, testes development in males remained elevated (and GSIs were non-significantly different, $p > 0.05$) in both January (2010 and 2011) and February (2009), suggesting that male *G. ceylonensis* gonad development is more protracted than females. De Silva (1991) observed protracted activity in the reproductive cycle of *G. ceylonensis*, but the present study confirms that this may be a life history aspect of males. Prolonged and aseasonal reproductive investment is shown in male tinfoil barb *Puntius schwanenfeldii* (McAdam et al. 1999). It is linked with increasing probability of spawning success that ensures eggs are fertilized throughout the spawning season and assures maximum spawning is successful in population recruitment. Generally, the condition of gonads in mature male fish is maintained at a higher level for longer periods than females (Le Cren 1951). Earlier studies on reproductive physiology of *Garra* spp. have shown asynchronous gonad development between sexes, namely *G. ceylonensis* (De Silva 1991), and *G. rufa* (Patimar et al. 2010; Abedi et al. 2011), but earlier maturity of males of the latter (Patimar et al. 2010; Abedi et al. 2011).

Abou-Seedo and Dadzie (2004) hypothesized that larger body size in females may increase capacity for egg production but the larger body size of female *G. ceylonensis* in intense agricultural sites in the Uma-oya was associated with sub-optimum fecundity and correspondingly lower gonad size relative to fish from low contaminated sites. The failure to show increases in gonad size with increases in growth rate and improved energy storage (liver size, condition) has been interpreted as an indication of metabolic disruption (Munkittrick et al. 1991; Parrott 2005).

Higher fecundity (total number of eggs/fish) together with higher gonad biomass (i.e. high GSI) in lower elevation females during the prespawning season indicates an eutrophication response to increased food availability (Munkittrick et al. 2000). Sub-optimum gonad investment and fecundity of females at sites associated with high agricultural inputs may be due to impaired resource partitioning. Although the fish obtained sufficient resources for improving their body

size, they were unable to convert that food to gonadal development (Munkittrick et al. 2000, 2002).

Prespawning condition (K) of female *G. ceylonensis* were not significantly ($p > 0.05$) different between sites in the agricultural catchment, and more interestingly between reference and agricultural sites. Traditionally, population level impact assessment (e.g. Environmental Effects Monitoring program in Canada (Environment Canada 2010) on fish body condition sets a critical effect size of 10% difference relative to that of a reference population. The impact level of body condition (i.e. 10%) is more restrained than the levels (i.e. 25%) that are used for GSI, LSI and fecundity parameters (Munkittrick et al. 2002; Lowell et al. 2003; Munkittrick et al. 2009).

Carriquiriborde et al. (2007) observed a protective capacity of pesticides (viz. without significant change in condition) on aquatic organisms that has been augmented by suspended particulate matter and sediment under field conditions. Chiang et al. (2011) observed a lower variability of body condition among sites not more than 7% in resident fish species (female *Trichomycterus areolatus*) exposed to effluent gradients of pulp and paper mills in the Itata River Basin in central Chile. Another study in the USA, Irons et al. (2007) documented that the decline of fish body condition, as low as 5 to 7% was influential in overall population health caused by food limitations of non-native fish species on native fish species, such as bigmouth buffalo (*Ictiobus cyprinellus*), and gizzard shad (*Dorosoma cepedianum*). However, fish condition has been shown to be rather insensitive for assessing effects between impacted (urban and agricultural activities) and reference streams, despite significant differences in growth, reproduction (Fitzgerald et al. 1999; Gray et al. 2005), and physiological impairment (de la Torre et al. 2005; Wijeyaratne and Pathiratne 2006; Carriquiriborde et al. 2007).

The measurements of length-weight relationships and condition factor underline the importance of understanding the environmental and biological effects on growth and

reproductive success in fish. For instance, the condition factor (K) and b value of the length-weight relationship in fish reflects information on the physiological state of the fish in relation to its welfare (Le Cren 1951). The value of $b < 3$ in adults revealed changes in body shape during the periods of prespawning of *G. ceylonensis* primarily at mid-stream sites in the Uma-oya. This difference in the b value suggests that the scaling coefficient (b) of length-weight relationship is a useful parameter to reflect anthropogenic stress on *G. ceylonensis*. Gray et al. (2002) studied the usefulness of non-lethal measurements, such as length and weight for impact studies. They found that there were differences in the size distribution and growth of slimy sculpin (*Cottus cognatus*) residing in streams influenced by agricultural inputs. However, it appears that the scaling coefficient (b), but not the condition (K) of fish (though both integrating length and weight parameters) would provide useful information of anthropogenic impact (Baby et al. 2011), and therefore would be useful for studies with minimal lethal sampling (Gray et al. 2002). Improved fish condition assumes that heavier fish at a given length are in better condition, and therefore nutritionally nourished and healthy (Chellappa et al. 1995; Jones et al. 1999; Eastwood and Couture 2002). Prolonged or severe stress (e.g. chronic exposure to a pollutant) will cause resident fish species to alter their energy allocation patterns and hence alter metabolism, growth or reproduction patterns. The negative allometric growth (lower b-values) represents fish under stress (Eastwood and Couture 2002), except for adaptive reasons in special circumstances, such as for burrowing lifestyle of fish species (e.g. *Cobitis narentana*) (Zanella et al. 2003),. Growth trajectory of natural populations of *Garra* spp. have shown to be isometric ($b = 3$) associated with "heavy-bodied cyprinid forms" by some studies (Sani et al. 2010; Baby et al. 2011; Birecikligil and Çiçek 2011).

4.5.3 Implications for future monitoring

The measurable impacts on fish in receiving environments range from nutrient enrichment to metabolic disruption (Lowell et al. 2004). The LSI data for female *Garra*

collected from two stream catchments provided important information about the patterns of energy utilization during prespawning periods. During the prespawning periods, both female and male *G. ceylonensis* in the Uma-oya sites showed larger livers (LSI) and higher condition (K) compared to post-spawning periods, although male livers were persistently larger through spawning periods. Increased liver size and larger gonads during the prespawning periods of *G. ceylonensis* indicate active feeding and accumulation of energy reserves for spawning (Baby et al. 2011). Similar observations have been made for nutrient enrichment in streams below pulp and paper mill effluent discharge (Munkittrick et al. 1991; Munkittrick et al. 1994; Wartman et al. 2009), municipal and sewage outfalls (Chambers et al. 2000; Galloway et al. 2003), and agricultural runoff (Bergamaschi et al. 2001; Gray et al. 2005; Gray and Munkittrick 2005). There is a large reduction in female *G. ceylonensis* liver (by almost one-quarter) and gonad size (by almost one-half) between January and February. Liversomatic index and GSI have been widely used for determination of the balance in energy utilization, where Litaay and De Silva (2003) have shown inverse trends between GSI and LSI. Gray et al. (2005) explained that concurrent declining of liver and gonad during spawning times would coincide with vitellogenesis in female sculpin (*Cottus cognatus*), when the protein vitellogenin produced in the liver is transferred and incorporated into the developing oocytes as yolk proteins in granular or platelet form in the ooplasm.

Sumith et al. (2011) observed that *G. ceylonensis* populations in the Knuckles streams (in Sri Lanka) undergo spawning migrations up tributary streamlets (see also Chapter 3) which suggest that spawning occurs during high flow periods, and that the liver energy stores in female *Garra* in the Knuckles were fully utilized during prespawning migrations. However, liver energy status in the Uma-oya females during prespawning period in January was highest when the largest increase in gonad development occurred. A similar pattern of energy storage has been shown with fish species whose liver reserves continue to be higher during active feeding

(Galloway and Munkittrick 2006). This difference implies that spawning migrations in *Garra* populations in the Uma-oya may not be an important life history aspect as proposed by De Silva (1991). Similarly, Konswal (1990) reported that spawning migration is not a life history aspect of *G. gotyla gotyla* although it is with congeneric *G. pectinopterus*. This observation reiterates the importance of genetic assessments in order to understand the possibility of species divergence and associated mechanisms underlying the reproductive timing in *G. ceylonensis*.

Long-term trends in cyclical sediment fluxes (elevated levels of sand and silt fractions with high intensity of rainfall) were obvious during November through January in the Uma-oya catchment. De Silva (1991) hypothesized that higher turbidity could curtail sunlight penetration and photosynthetic production that disrupts preferred food (i.e. epilithic/ epiphytic algae and diatoms) production in streams. The Ceylon stone sucker (*G. ceylonensis*) is a small freshwater benthic fish species that inhabits lotic environments with fast flowing water and feeds on detritus, algal mats and periphyton attached to rock surfaces (Ojha and Singh 1992). In hill streams with low nutrient inputs, food limitation is reflected in variations in body and physiological conditions that reflect the ecological conditions.

The Uma-oya populations may be demonstrating reproductive adaptations to ensure better survival of offspring during times of ample food supplies by shifting away from periods of intensive sediment fluxes. Walters et al. (2003) showed that increasing fine sediment in streams has the largest negative effect on benthopelagic fish species affecting various life history characteristics including reduction of egg and fry survivorship, lowering prey densities through habitat destruction or increased drift. The brook trout (*Salvelinus fontinalis*), a substrate spawner adapted to streambed sediment deposition by restricting its spawning to locations with constricted flow and accelerated surface water velocities, i.e. locations where visible gravel substrates is seen in heavily sediment-impacted streams (Curry and MacNeill 2004). By considering life history characteristics such as substrate spawning and algal feeding, *G.*

ceylonensis has the potential to be seriously impacted by sediment loading in streams, among other stressors. Given extremely high seasonal rainfalls, exposure of this species to fine sediments is a natural occurrence, but sediment effects from intensive agricultural activities over the last few decades can be overwhelmingly influential. Seasonal shift in reproductive life history mode of tinfoil barb *Puntius schwanenfeldii* has been reported in response to river regulation in a comparatively shorter time scale (McAdam et al. 1999).

Reproductive activity in teleost fishes is guided by necessary cues from the environment, which influences endogenous physiological cues which synchronize their behaviour with that of conspecifics (Helfman et al. 1997). Konswal (1990) reported a possible role of turbidity as a spawning cue in *Garra* sp. in a tropical stream. The turbidity measure is an index of light scattering by suspended particles (Davies-Colley and Smith 2001) which does not reflect the nature of the suspended material which could include algal cells, dissolved humic substances and minerals and organic matter (Bilotta and Brazier 2008). Ng and Munro (1997) showed that water conductivity was influential on reproductive synchronization and the final maturation and spawning in several cyprinids. Such a situation indicates the significance of environmental changes that act as regulators of gonadal development. In fact, components mixed into a turbidity matrix might have a positive influence on fish reproduction, and high suspended sediment concentrations have physical attributes that can have undesirable effects on fish. The cyclical peak sediment fluxes (flow, sand and silt fractions) demand high energetic costs for behavioral and reproductive performance in fish. As a consequence, the observed adaptations may be applicable across regions, where sediment impacts are persistently present. De Silva (1991) reported dry-season spawning during January-February in an alternate stream, Sarasavi-oya in the upper Mahaweli River catchment. Adults may not be the most sensitive stage to sediment concentration and the young-of-the-year (YoY) can be compromised by increased foraging costs and reduced feeding efficiency (Zamor and Grossman 2007; Hazelton and

Grossman 2009) and also by direct damage to gill epithelia (Sutherland and Meyer 2007). Available evidence suggests that most fish species are reproductively programmed to spawn during periods of high food availability for early larval stages (Lasker 1981). According to Helfman et al. (1997), dry season spawners tend to give higher levels of parental care and young provisioning. However, this is not true in *G. ceylonensis* where parental care or young provisioning has not been observed (De Silva 1991). However, it remains open to discussion whether the observed situation reflects another seasonality effect, or as a result of genetic diversity.

4.6 Conclusion

This is the first study of using *G. ceylonensis* as a sentinel monitoring species to assess agricultural impacts in tropical stream systems. There are challenges in finding suitable reference locations in agricultural catchments, and for the Sri Lankan situation, we proposed a cluster gradient design (Chapter 2; Sumith and Munkittrick 2011a). However, an incomplete understanding of the biology of *G. ceylonensis* in different geographies hinders reference site selection and in the understanding the natural variability within this species on a broader spatial scale. Prespawning *Garra* sp. in the agricultural sites have larger livers and body size relative to populations in the Knuckles reference streams suggesting nutrient enrichment in the agricultural areas. However, the absence of concomitant increases in gonad size suggests some limitations in fish health performance in agricultural areas. A series of iterative (i.e. adaptive) studies may be required to progressively isolate the causative agents responsible for differential response of performance in *G. ceylonensis*.

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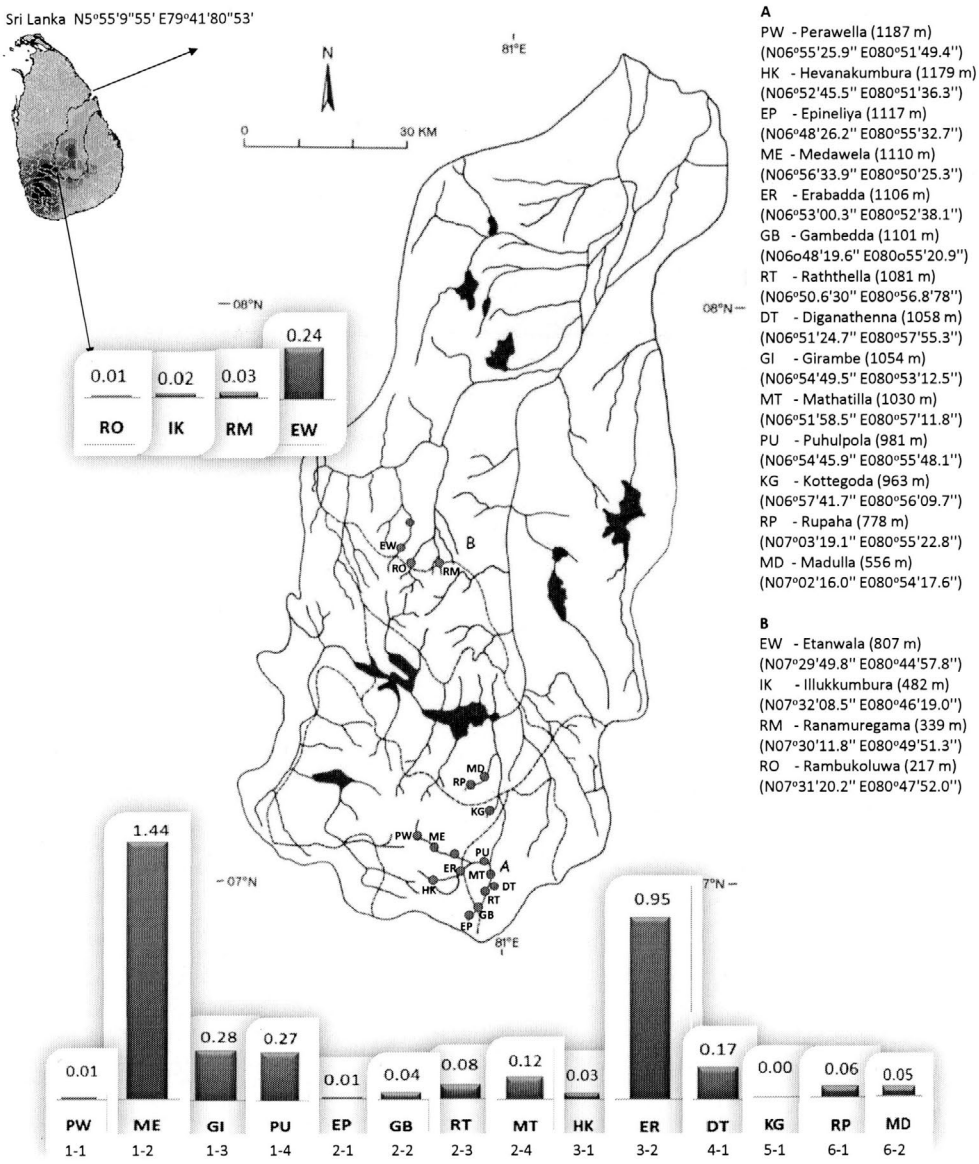

Figure 4-1. Distribution of sampling sites in the Mahaweli River basin; (A) Uma-oya catchment (B) Amban-ganga catchment in the Knuckles; Sampling sites appear as a closed circle. Bar charts show density (number/m^2) of *G. ceylonensis*.

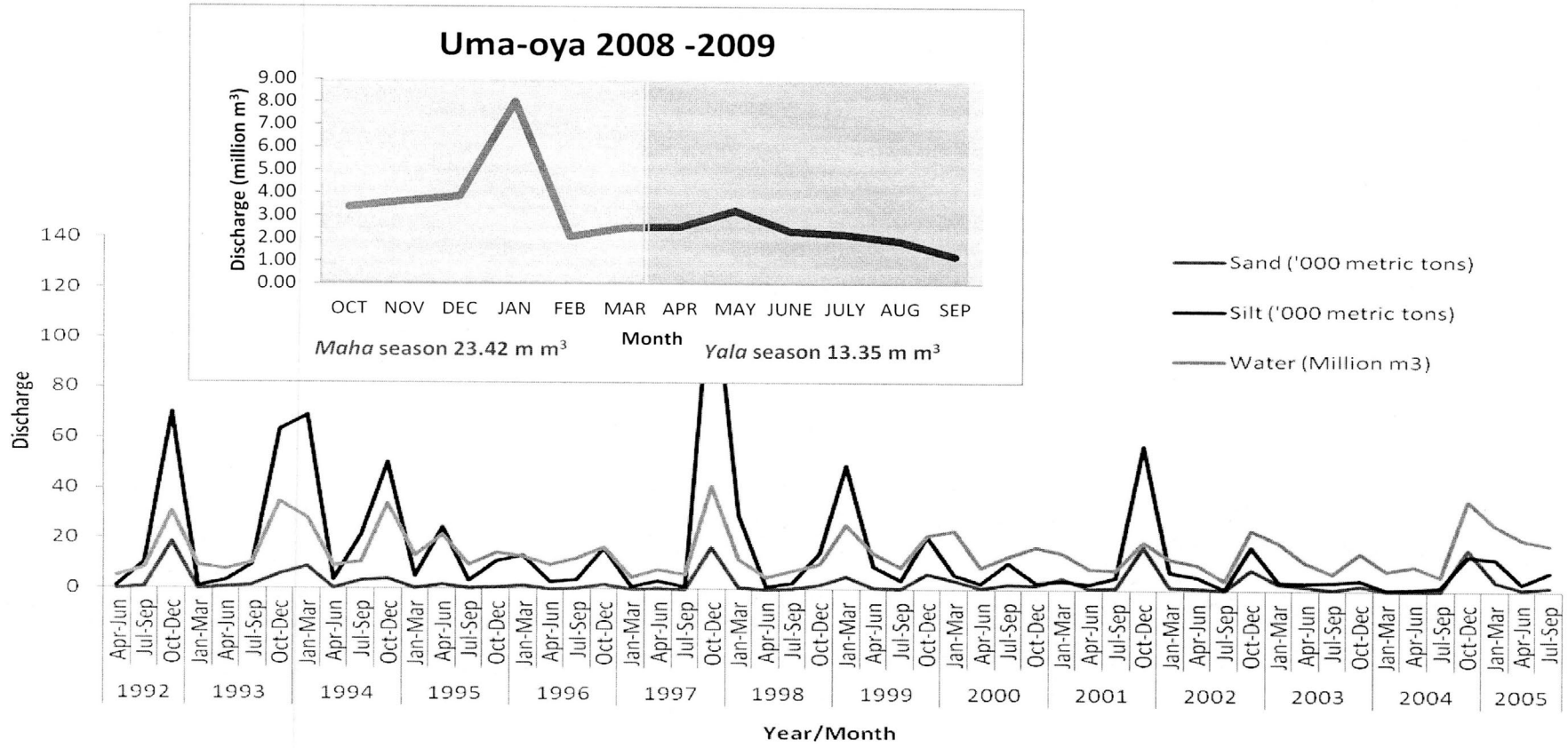

Figure 4-2. Quarterly discharge dynamics of sand, silt (as sediment simulates) and water in the Uma-oya rated at the gauging station located at Welimada during 1992 and 2005. *Insert*: Monthly discharge dynamic in the Uma-oya; Reference year 2008 and 2009 (Mahaweli Authority of Sri Lanka). The representative catchment extent: 9834.67 ha.

A

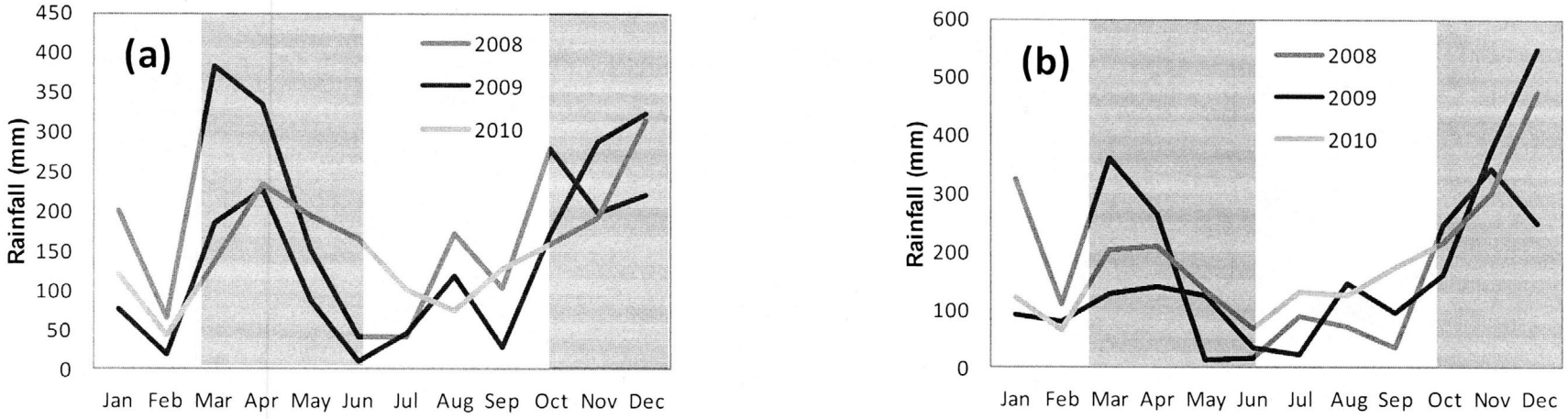

Figure 4-3 (**A**). Typical bimodal rainfall pattern (average of 6 data collection stations) in the study catchments (a) Uma-oya catchment 3-year mean ± SD 1868 ± 306 mm, *Yala* season mean ± SD 178 ± 50 mm, *Maha* season mean ± SD 211 ± 14 mm, (b) Knuckles catchment 3-year mean ± SD 2083 ± 142 mm, *Yala* season mean ± SD 140 ± 30 mm, *Maha* season mean ± SD 287 ± 8 mm. Shaded areas show two monsoonal periods, left: Southwest monsoon, right: Northeast monsoon.

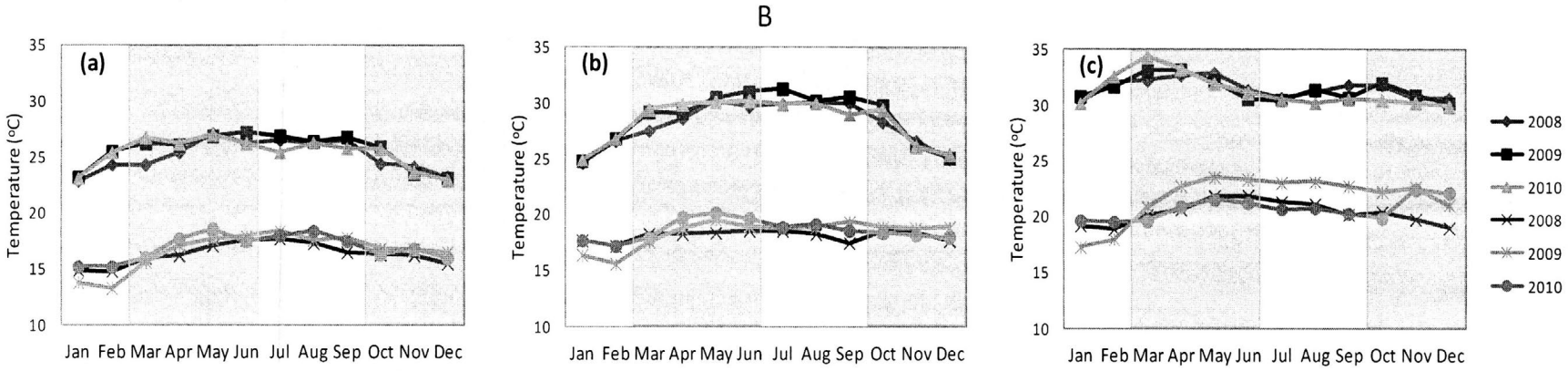

Figure 4-3 (**B**). Yearly pattern of temperature (minimum and maximum) in the study catchments (a) Uma-oya (upper catchment), (b) Uma-oya (lower catchment), (c) Knuckles catchment. Shaded areas show two monsoonal periods, left: Southwest monsoon, right: Northeast monsoon.

C

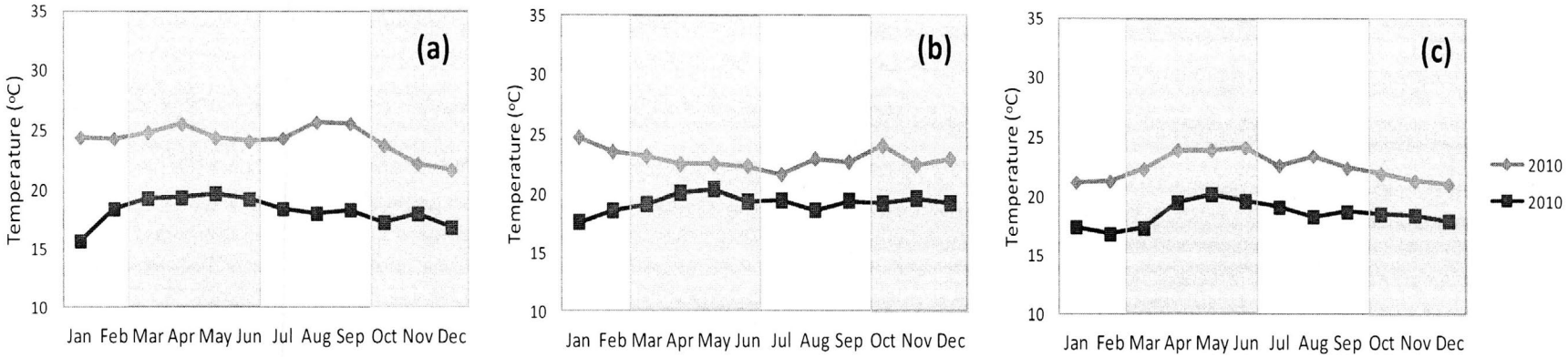

Figure 4-3 (**C**). Pattern of stream water temperature during 2010 (monthly minimum and maximum). (a) Uma-oya (upper catchment); maximum mean ± SD 22.9°C ± 0.8, minimum mean ± SD 19.2°C ± 0.7 as measured in Perawella (1,187 m above MSL), (b) Uma-oya (lower catchment), Maximum mean ± SD 24.2°C ± 1.2, Minimum mean ± SD 18.2°C ± 1.2 as measured in Welimada (1,078 m above MSL), (c) Knuckles catchment, maximum mean ± SD 22.4°C ± 1.1, minimum mean ± SD 18.5°C ± 1.0 as measured in Etanwela (807 m above MSL). Shaded areas show two monsoonal periods, left: Southwest monsoon, right: Northeast monsoon.

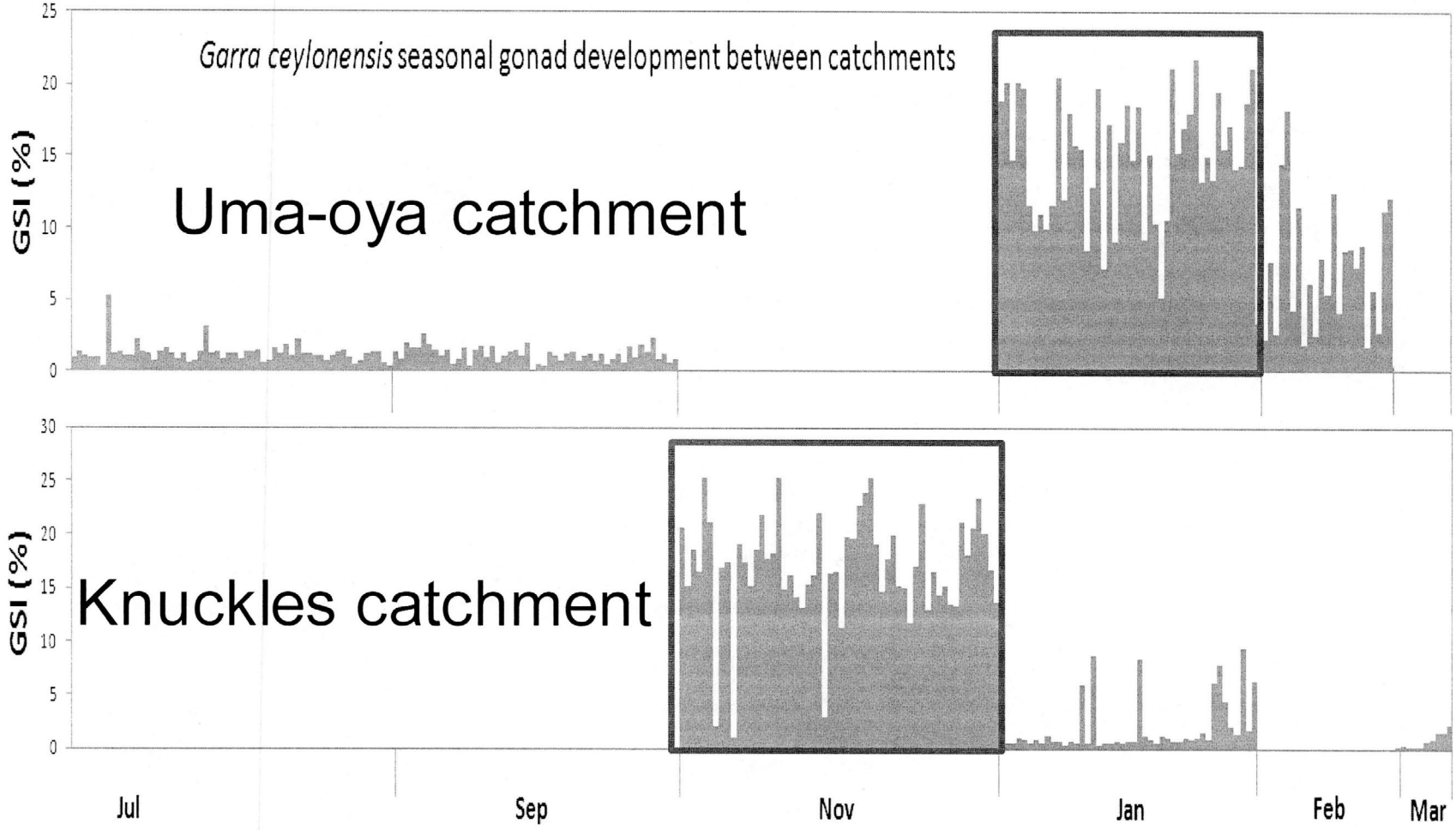

Figure 4-4. Seasonal gonad development (GSI%) of *Garra ceylonensis* in the Uma-oya catchment and the Knuckles streams.

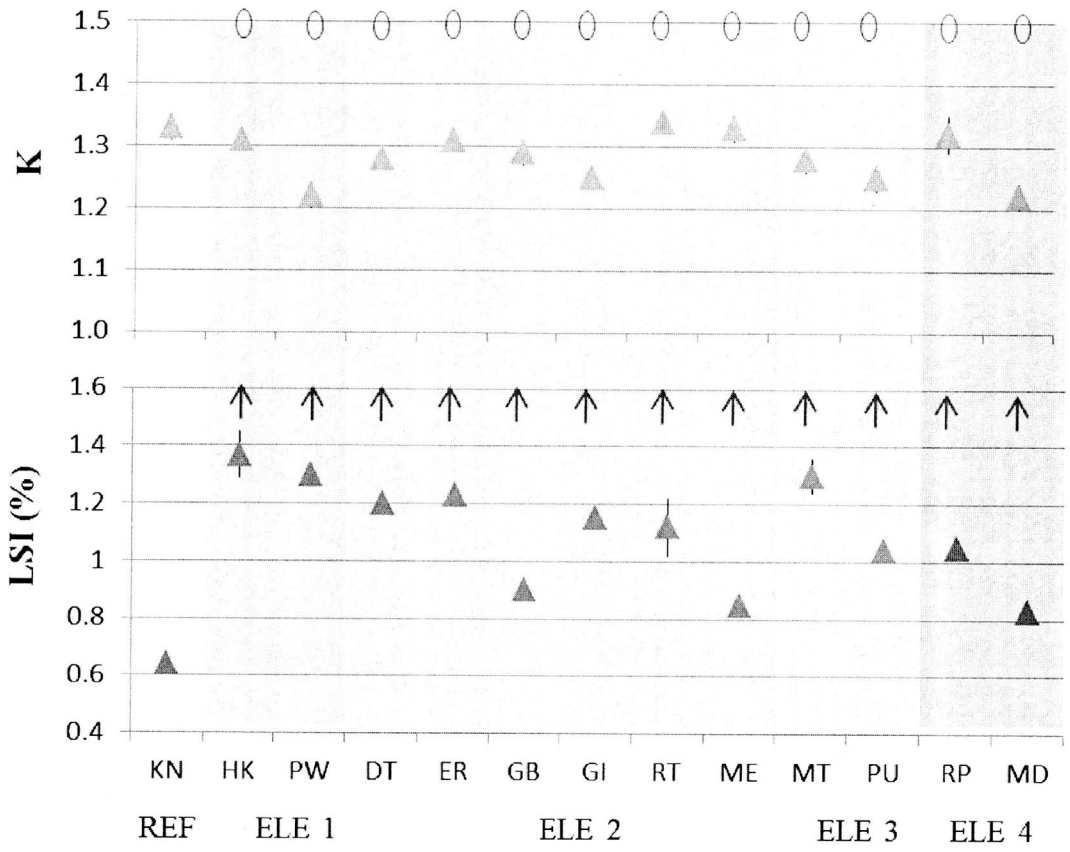

Figure 4-5. Pattern of population response parameters (K and LSI) in female *G. ceylonensis* in the Uma-oya catchment in comparison to the Knuckles reference population. Data Mean ± SE; ecologically relevant responses in fish were counted as 10 and 25% deviation from mean reference values of K and LSI, respectively: increase (↑), decrease (↓), no response (O). REF = Reference sites; ELE 1 = Elevation category 1 (> 1,150 m); ELE 2 = Elevation category 2 (> 1,050 < 1,150 m); ELE 3 = Elevation category 3 (> 950 < 1,050 m) and ELE 4 = Elevation category 4 = < 950 m above MSL. Fulton's Condition (K) = [(weight/length^3) *100], Liversomatic index (LSI) = [(liver weight/body weight)*100]. For descriptive site codes, see Figure 4-1 legend.

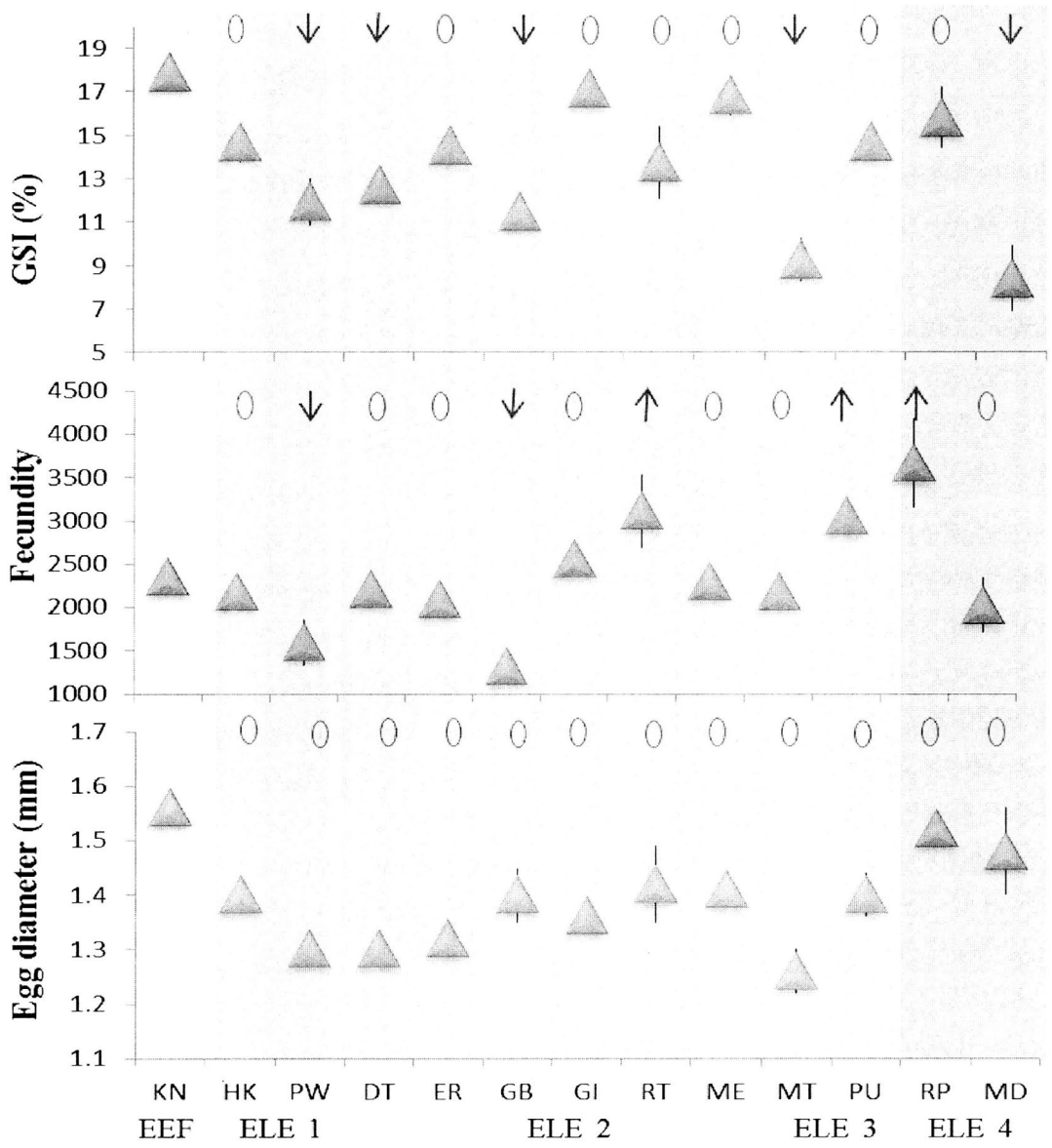

Figure 4-6. Pattern of reproductive response parameters (GSI, fecundity and egg diameter) in female *G. ceylonensis* in the Uma-oya catchment in comparison to the Knuckles reference population. Data Mean ± SE; ecologically relevant responses in fish were counted as 25% deviation from mean reference values: increase (↑), decrease (↓), no response (O). REF = Reference sites; ELE 1 = Elevation category 1 (> 1,150 m); ELE 2 = Elevation category 2 (> 1,050 < 1,150 m); ELE 3 = Elevation category 3 (> 950 < 1,050 m) and ELE 4 = Elevation category 4 = < 950 m above MSL. Gonadosomatic index (GSI) = [(gonad weight/body weight)*100]. For descriptive site codes, see Figure 4-1 legend.

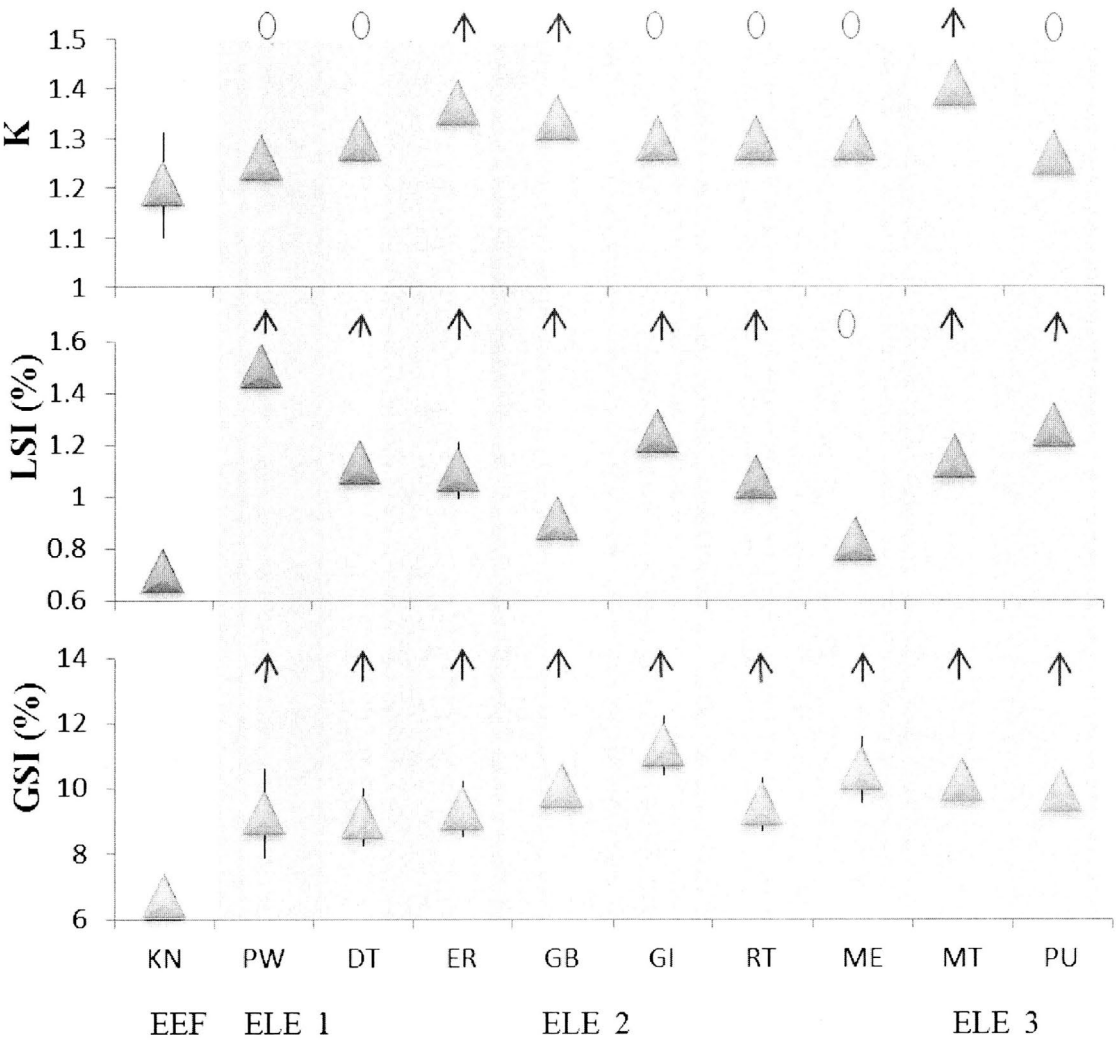

Figure 4-7. Pattern of population response parameters (K, LSI and GSI) in male *G. ceylonensis* in the Uma-oya catchment in comparison to the Knuckles reference population. Data Mean ± SE; ecologically relevant responses in fish were counted as 10 deviation from mean reference values of K and 25% deviation of LSI and GSI: increase (↑), decrease (↓), no response (O). REF = Reference sites; ELE 1 = Elevation category 1 (> 1,150 m); ELE 2 = Elevation category 2 (> 1,050 < 1,150 m); ELE 3 = Elevation category 3 (> 950 < 1,050 m) and ELE 4 = Elevation category 4 = < 950 m above MSL. Fulton's Condition (K) = [(weight/length^3) *100], Liversomatic index (LSI) = [(liver weight/body weight)*100]. Gonadosomatic index (GSI) = [(gonad weight/body weight)*100]. For descriptive site codes, see Figure 4-1 legend.

Table 4-1. Water quality indicators in stream sections surveyed for fish in the Uma-oya (agricultural catchment) and Knuckles streams (forested catchment) during 2009 and 2011. Values are mean ± SD. Units are in mg l^{-1}, except for conductivity in µS cm^{-1}, chlorophyll-*a* in mg cm^{-2}.

Parameter[†]	Site[1]						
	Agric	Agric	Forested	Agric	Forested	Agric	Agric
	2009 Jul (n = 5)	2010 Jan (n = 6)	2010 Jan (n = 4)	2010 Apr (n = 8)	2010 Apr (n = 4)	2010 Oct (n = 14)	2011 Jan (n = 14)
pH	7.37 ± 0.11	7.24 ± 0.16	6.58 ± 0.22	7.62 ± 0.25	7.18 ± 0.43	6.92 ± 0.23	6.88 ± 0.25
Conductivity	193.4 ± 45.7	112.0 ± 21.8	30.3 ± 8.3	156.9 ± 39.0	33.0 ± 3.7	118.7 ± 22.3	112.0 ± 25.3
Nitrate-N	0.77 ± 0.46	1.12 ± 0.45	0.37 ± 0.15	0.86 ± 0.55	0.27 ± 0.04	1.39 ± 1.02	1.91 ± 1.06
Sulphate	7.96 ± 3.01	3.43 ± 1.28	1.93 ± 1.13	4.32 ± 2.14	2.42 ± 0.92	3.22 ± 1.53	4.38 ± 2.97
Total alkalinity	91.0 ± 12.4	37.8 ± 6.9	19.7 ± 6.9	114.8 ± 23.7	20.9 ± 3.7	52.7 ± 10.2	25.3 ± 7.0
Total hardness	95.2 ± 19.1	37.9 ± 7.5	16.2 ± 3.4	39.2 ± 10.9	16.0 ± 4.7	57.7 ± 10.0	47.2 ± 14.1
Total suspended solids (TSS)	88.2 ± 54.0	14.5 ± 9.9	3.3 ± 1.3	15.4 ± 7.7	8.5 ± 3.1	18.0 ± 8.2	26.4 ± 21.6
Total nitrogen	7.59 ± 2.56	8.62 ± 2.92	0.55 ± 0.29	6.40 ± 4.11	1.35 ± 0.65	2.43 ± 2.25	2.49 ± 1.24
Total ammonia	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.22 ± 0.02	0.22 ± 0.04	0.16 ± 0.14	0.11 ± 0.10
Total phosphate	0.24 ± 0.18	0.18 ± 0.06	0.08 ± 0.02	0.22 ± 0.13	0.09 ± 0.04	0.14 ± 0.14	0.12 ± 0.10
Total organic carbon (TOC)	9.93 ± 4.81	4.63 ± 2.37	1.04 ± 0.59	10.60 ± 5.79	1.76 ± 0.77	4.34 ± 3.19	6.97 ± 2.60
Biological Oxygen Demand (BOD)	3.26 ± 0.96	1.29 ± 0.43	1.46 ± 0.60	1.94 ± 1.16	1.02 ± 0.51	1.28 ± 0.49	0.81 ± 0.36
Chemical Oxygen Demand (COD)	29.20 ± 3.70	7.27 ± 2.12	3.40 ± 1.38	11.50 ± 8.17	3.69 ± 2.39	7.70 ± 3.78	11.10 ± 5.22
Chlorophyll-*a*	3.12 ± 2.79	1.53 ± 1.78	1.33 ± 0.41	3.94 ± 3.61	5.31 ± 3.38	0.99 ± 0.96	0.86 ± 0.88

[†]Each sample was replicated 3 times and averaged before the assessment.

Table 4-2. Seasonal summary statistics for *Garra ceylonensis* sampled in the Uma-oya by sex.

Sex	Month	n	Length (cm)	Weight (g)	K (%)	LSI (%)	GSI (%)[†]
Female	Feb 2009	24	12.27 ± 0.47^{a}	24.05 ± 2.94^{a}	1.17 ± 0.03^{b}	0.91 ± 0.13^{b}	7.11 ± 0.91^{b}
	Jul 2009	119	13.10 ± 0.19^{a}	28.79 ± 1.26^{a}	1.21 ± 0.01^{b}	1.05 ± 0.03^{b}	1.57 ± 0.29^{c}
	Sep 2009	115	12.48 ± 0.14^{a}	23.85 ± 0.75^{a}	1.20 ± 0.01^{b}	1.04 ± 0.02^{b}	1.15 ± 0.06^{c}
	Jan 2010	278	11.59 ± 0.10^{b}	20.88 ± 0.56^{b}	1.28 ± 0.01^{a}	1.24 ± 0.03^{a}	14.31 ± 0.41^{a}
	Jan 2011	335	11.62 ± 0.11^{b}	22.03 ± 0.65^{b}	1.30 ± 0.01^{a}	1.27 ± 0.04^{a}	13.85 ± 0.35^{a}
Male	Feb 2009	18	9.13 ± 0.32^{c}	8.60 ± 0.77^{c}	1.08 ± 0.02^{c}	1.26 ± 0.11^{b}	7.41 ± 0.78^{a}
	Jul 2009	94	11.62 ± 0.20^{a}	20.63 ± 1.20^{a}	1.24 ± 0.01^{b}	1.03 ± 0.04^{c}	1.31 ± 0.21^{b}
	Sep 2009	66	11.40 ± 0.18^{ab}	18.63 ± 0.77^{a}	1.22 ± 0.02^{b}	1.11 ± 0.02^{bc}	1.23 ± 0.12^{b}
	Jan 2010	64	10.78 ± 0.21^{b}	17.29 ± 1.00^{a}	1.30 ± 0.01^{a}	1.25 ± 0.05^{b}	10.41 ± 0.42^{a}
	Jan 2011	207	9.73 ± 0.12^{c}	13.20 ± 0.50^{b}	1.33 ± 0.01^{a}	1.45 ± 0.05^{a}	9.88 ± 0.25^{a}

Values are mean ± SE, means within a column that do not share a letter are significantly different between seasons within a sex (ANOVA, $p < 0.001$). gonadosomatic index, GSI (gonad weight/body weight)*100), liversomatic index, LSI (liver weight/body weight)*100) and condition factor, K [(weight/length^3) *100], n = number of fish (sample size), [†]GSI > 1% represents adults

Table 4-3. Young-of-the-Year (YoY) size distribution in *Garra ceylonensis* in Uma-oya stream locations.

Site	YoY size class (cm)	Percent (%) in the sampling period		
		February (2009)	July (2009)	September (2009)
Girambe	<9.8	55.5	23.5	14.1
	<10.3	62.9	38.2	14.9
Erabadda	<9.8	50.7	30.9	40.4
	<10.3	62.7	43.2	41.2
Medawela	<9.8	62.1	66.8	53.1
	<10.3	71.7	71.9	56.9

Table 4-4. Summary statistics for male and female *Garra ceylonensis* captured in the Uma-oya mainstream and tributary streams on contamination and elevation scales during January, 2010 and 2011. Values are mean ± SE. Values sharing a lowercase superscript letters are not significantly different (ANOVA, $p > 0.05$).

Sex	Scale	n	Parameter				
			L (cm)	W (g)	GSI (%)	LSI (%)	K (%)
Female	Contamination scale						
	1-High	109	12.80 ± 0.21^{a}	28.61 ± 1.35^{a}	11.66 ± 0.65^{b}	1.19 ± 0.04^{a}	1.27 ± 0.01^{a}
	2-Medium	339	11.27 ± 0.09^{b}	19.53 ± 0.49^{b}	15.23 ± 0.37^{a}	1.07 ± 0.02^{b}	1.30 ± 0.01^{a}
	3-Low	165	11.51 ± 0.13^{b}	20.89 ± 0.77^{b}	13.25 ± 0.43^{a}	1.23 ± 0.03^{a}	1.29 ± 0.01^{a}
	Elevation scale[†]						
	>1,150	48	11.20 ± 0.23^{b}	19.45 ± 1.21^{b}	13.81 ± 0.73^{a}	1.35 ± 0.06^{a}	1.30 ± 0.01^{a}
	>1,050<1,150	430	11.24 ± 0.08^{b}	19.33 ± 0.42^{b}	14.82 ± 0.32^{a}	1.11 ± 0.02^{bc}	1.29 ± 0.01^{a}
	>950<1,050	109	12.80 ± 0.21^{a}	28.61 ± 1.35^{a}	11.66 ± 0.65^{b}	1.19 ± 0.04^{ab}	1.27 ± 0.01^{a}
	<950	26	13.34 ± 0.33^{a}	31.64 ± 2.47^{a}	12.09 ± 1.24^{ab}	0.94 ± 0.03^{c}	1.27 ± 0.02^{a}
Male	Contamination scale						
	1-High	118	10.47 ± 0.18^{a}	16.61 ± 0.85^{a}	10.05 ± 0.30^{a}	1.22 ± 0.03^{a}	1.33 ± 0.01^{a}
	2-Medium	103	9.79 ± 0.13^{b}	12.98 ± 0.50^{b}	10.00 ± 0.37^{a}	1.00 ± 0.03^{b}	1.32 ± 0.01^{a}
	3-Low	50	9.19 ± 0.21^{b}	10.85 ± 0.71^{c}	9.91 ± 0.55^{a}	1.25 ± 0.05^{a}	1.30 ± 0.02^{a}
	Elevation scale						
	>1,150	22	8.67 ± 0.40^{b}	9.62 ± 1.43^{c}	10.42 ± 0.94^{a}	1.43 ± 0.08^{a}	1.29 ± 0.02^{a}
	>1,050<1,150	124	9.71 ± 0.11^{a}	12.56 ± 0.40^{b}	9.88 ± 0.34^{a}	1.03 ± 0.03^{c}	1.32 ± 0.01^{a}
	>950<1,050	118	10.47 ± 0.18^{a}	16.61 ± 0.85^{a}	10.05 ± 0.30^{a}	1.22 ± 0.03^{ab}	1.33 ± 0.01^{a}
	<950	7	10.46 ± 0.68^{a}	15.66 ± 3.01^{ab}	10.19 ± 0.72^{a}	0.96 ± 0.08^{bc}	1.28 ± 0.03^{a}

Gonadosomatic index, GSI (gonad weight/body weight)*100), liversomatic index, LSI (liver weight/body weight)*100) and condition factor, K [(weight/length^{3}) *100], n = Number of fish [†]Elevation m above MSL

Table 4-5. Summary statistics on fecundity parameters of female *Garra ceylonensis* captured in the Uma-oya mainstream and tributary streams on contamination scale and elevation scales during January 2010 and 2011. Values are mean ± SE. Values sharing a lowercase superscript letters are not significantly different (ANOVA, $p > 0.05$).

Scale	n	Fecundity	Egg weight (µg)	Egg diameter (mm)
Contamination scale				
1-High	100	2614 ± 140^{a}	2.23 ± 0.72^{a}	1.33 ± 0.03^{a}
2-Medium	307	2249 ± 69^{b}	1.64 ± 0.04^{a}	1.36 ± 0.01^{a}
3-Low	157	2245 ± 97^{ab}	1.39 ± 0.04^{a}	1.34 ± 0.02^{a}
Elevation scale				
>1,150	48	1953 ± 131^{b}	1.47 ± 0.07^{a}	1.35 ± 0.02^{ab}
>1,050<1,150	394	2241 ± 60^{b}	1.56 ± 0.04^{a}	1.34 ± 0.01^{b}
>950<1,050	100	2614 ± 140^{a}	2.23 ± 0.72^{a}	1.33 ± 0.03^{b}
<950	21	3038 ± 377^{a}	1.61 ± 0.09^{a}	1.50 ± 0.03^{a}

Table 4-6. Relationship between total weight (W) and total length (L) of *Garra ceylonensis* in the Uma-oya sites compared to the Knuckles streams. n, sample size; r^2, correlation coefficient; b, allometric coefficient.

Site[†]	n	Equation	Slope (b)	r^2	Relationship (*t*-test)
RT	43	Log W = - Log 1.99 + 3.10 Log L	3.10	0.980 ($p < 0.0001$)	Isometry
RP	18	Log W = - Log 1.93 + 3.04 Log L	3.04	0.988 ($p < 0.0001$)	Isometry
PW	49	Log W = - Log 1.74 + 2.83 Log L	2.83	0.984 ($p < 0.0001$)	Allometry (-)
PU	211	Log W = - Log 1.73 + 2.83 Log L	2.83	0.961 ($p < 0.0001$)	Allometry (-)
MT	107	Log W = - Log 1.67 + 2.79 Log L	2.79	0.963 ($p < 0.0001$)	Allometry (-)
ME	188	Log W = - Log 1.52 + 2.64 Log L	2.64	0.903 ($p < 0.0001$)	Allometry (-)
MD	15	Log W = - Log 1.56 + 2.67 Log L	2.67	0.946 ($p < 0.0001$)	Allometry (-)
HK	42	Log W = - Log 1.90 + 3.01 Log L	3.01	0.965 ($p < 0.0001$)	Isometry
GI	194	Log W = - Log 1.69 + 2.80 Log L	2.80	0.941 ($p < 0.0001$)	Allometry (-)
GB	48	Log W = - Log 1.70 + 2.82 Log L	2.82	0.976 ($p < 0.0001$)	Allometry (-)
ER	236	Log W = - Log 1.61 + 2.73 Log L	2.73	0.947 ($p < 0.0001$)	Allometry (-)
EP	24	Log W = - Log 1.78 + 2.93 Log L	2.93	0.985 ($p < 0.0001$)	Isometry
DT	123	Log W = - Log 1.69 + 2.81 Log L	2.81	0.925 ($p < 0.0001$)	Allometry (-)
KN	79	Log W = - Log 1.93 + 3.03 Log L	3.03	0.934 ($p < 0.0001$)	Isometry

[†]For descriptive site codes, see Figure 4-1 legend.

Table 4-7. Some variable indices of *Garra ceylonensis* in the Uma-oya and the Knuckles streams in January 2010 (For descriptive site codes, see Figure 4-1 legend).

Variable	Uma-oya stream						Knuckles streams		Significance
	PU (n = 32,25)♥	DT (n = 4,62)	ER (n = 3,60)	GI (n = 6,42)	MT (n = 9,33)	ME (n = 10,56)	IK (n = 4,29)	RO (n = 2,8)	
Sex ratio (male: female)	1.28*	0.06*†	0.05*†	0.14*†	0.27†	0.18*†	0.14*†	0.25†	* $p < 0.01$ † $p < 0.0001$
Density♣ (individuals/ 100 m^2)	29 (26-36)	30	111 (50-226)	28 (12-53)	14	163 (73-253)	8 (1-24)		Kruskal-Wallis $p = 0.003$
Biomass (g/m^2)	5.7‡ ‡‡	3.4‡ ‡‡	16.9‡‡	4.7‡ ‡‡	2.2‡ ‡‡	16.4‡‡	0.1‡	0.3‡	‡ $p < 0.0001$ ‡‡ $p = 0.032$

♥ Within brackets (n) represent the number of male and female fish, respectively

* Significant difference from mean sex ratio of 0.54 (De Silva 1991)

† Significant difference from expected sex ratio of 1

‡ Significant difference from mean biomass 23.7 g m^{-2} (De Silva 1991)

‡‡ Significant difference from mean biomass 0.2 g m^{-2} of the Knuckles streams

♣ Mean of pooled sampling; minimum and maximum values are given within brackets unless recorded once.

CHAPTER 5

5 Seasonal exposure of fish to neurotoxic pesticides in an intensive agricultural catchment, Uma-oya, Sri Lanka: linking contamination and acetylcholinesterase inhibition [6]

5.1 Abstract

In monsoon tropics, agriculture primarily depends on the rainfall pattern. The annual cultivation pattern in the Uma-oya catchment in Sri Lanka is characterized by *Yala* and *Maha* rainfall periods and associated cropping. Two cultivation seasons were compared for pesticide residues: base flow, field drainage and, the runoff and supplementary sediment residue data for three sites in the catchment. Organophosphate and N-methyl carbamate pesticide analysis confirmed a higher concentration in the *Yala* season with low-flow conditions. Acetylcholinesterase (AChE) activity was measured by standard spectrometry in the brain, muscle, and eye tissues of three freshwater cyprinid fishes, Ceylon stone sucker (*Garra ceylonensis*), giant danio (*Devario malabaricus*), and striped rasbora (*Rasbora daniconius*) from three study sites during months overlapping two seasons in 2010 (December) and 2011 (July). Baseline AChE data were measured from fish samples from a forested reserve in the Knuckles. A 73% inhibition in muscle AChE activity in *G. ceylonensis* was associated with intense pesticide exposure months in the *Yala* season. The AChE inhibition was over 70% in *G. ceylonensis* eyes in both *Yala* (i.e. 76%) and *Maha* (i.e. 72.5%) seasons indicates particular sensitivity of eye tissue to inhibitors. The less dramatic AChE inhibition in the eye tissues in *D.*

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malabaricus and *R. daniconius* in both seasons indicates exemplary protective capacity of muscle AChE in fish. The highest inhibition of AChE (up to 60% in brain and up to 56% in muscle AChE activity in *R. daniconius* and up to 47.8% in brain and up to 64.6% in muscle AChE activity in *D. malabaricus*) occurred during the *Yala* season. Tissue AChE activity and physiological activity in fish were correlated. The results collectively indicate that AChE is a consistent exposure biomarker for diffused contaminant exposure in agricultural catchments.

Keywords: Agricultural catchment, Acetylcholinesterase, Organophosphates, N-methyl carbamates, Cyprinid fish

5.2 Introduction

The use of pesticides in developing countries has increased as the scale of cropping has expanded (Ecobichon 2001a), and at the same time, pesticide use has been increasing without a major change in agricultural land use (Wilson 1999). Sri Lanka is endowed with monsoonal climates that favour seasonal shifts in cropping patterns. Pesticide input into associated aquatic ecosystems is driven by both hydrological events and agricultural practices. Contemporary pesticide classes such as organophosphates (OPs) and N-methyl carbamates (NMCs) are short-lived in the environment, and their use has increased markedly in developing countries after the banning of highly persistent organochlorines (Aktar et al. 2009). The major distinction between the two classes of insecticides is the duration of acetylcholinesterase (AChE; EC 3.1.1.7) inhibition. Organophosphate-induced inhibition is effectively irreversible, whereas inhibition by NMC is reversible, which leads to a faster rate of recovery from enzyme inhibition (Ecobichon 2001b). In 2008, cholinesterase inhibitors such as OP and NMC insecticides made up almost 88% of the total amount of insecticides sold in Sri Lanka (Office of the Registrar of Pesticides, Sri Lanka).

These contemporary compounds degrade rapidly in the environment and require frequent application, thus increasing the cumulative environmental burden (Abdullah et al. 1997; Kookana and Simpson 2000), but they generally lack target specificity and have high acute toxicity toward many nontarget vertebrate and invertebrate species (Schulz and Liess 1999; Fulton and Key 2001). Some of the OPs and NMCs, such as chlorpyriphos, diazinon, and carbofuran, are detected more frequently in water bodies associated with agricultural fields, despite the rapid field dissipation and dilution of these insecticides (Aponso et al. 2003; Menike et al. 2010). Many of these pesticides degrade quickly under hot tropical climatic conditions (Laabs et al. 2002), and residue from most commonly used pesticides may not be detectable in water (Aponso et al. 2003).

Aquatic organisms are able to respond to contaminants even when such substances are not detectable in water or sediments (Sturn et al. 1999; de la Torre et al. 2005; Wijeyaratne and Pathiratne 2006; Chitmanat et al. 2008). Pesticides produce many physiological and biochemical changes in freshwater organisms by influencing the activities of several enzymes, and AChE inhibition has been instrumental as a sensitive biomarker of the effect of OP and NMC insecticides in aquatic ecosystems (Forget et al. 2003; Pathiratne et al. 2009; Pathiratne et al. 2010; Adedeji 2011). It has been suggested that AChE activity is highly conserved in organisms including fish (Bertrand et al. 2001), and environmental contaminants can up-regulate and down-regulate this enzymatic activity. From the standpoint of functionality, both effects are deleterious for fish survival (Ansari et al. 1987; Sandahl and Jenkins 2002; Dutta and Arends 2003; Golombieski et al. 2008). The inhibition of activity can also affect a variety of other life functions including growth, survival, feeding, and reproductive behavior of fish (Dutta and Arends 2003; Sandhal et al. 2005), leading to population-level effects (Baldwin et al. 2009). Acetylcholinesterase inhibition does not necessarily lead to the death of organisms but is useful

as an exposure biomarker for sub-lethal concentrations of contaminants (Fulton and Key 2001; van der Oost et al. 2003; Coelho et al. 2011; Nunes 2011).

Several studies have shown that fish inhabiting natural freshwater ecosystems may be affected by the intentional and unintentional spreading of pesticides (Gruber and Munn 1998; Wijeyaratne and Pathiratne 2006; Pathiratne et al. 2010). *In vivo* exposure of fish species to AChE inhibitors led to differential response patterns of AChE activity in different tissues (brain, muscle, plasma, liver, and eyes; Mdegela et al. 2010). Currently, qualitative differences in AChE enzymatic activities among fish species are largely associated with individual exposure conditions (Sandahl et al. 2005). Comparison of differential tissue responses to AChE inhibitors among multiple species in real agricultural environments is limited (Gruber and Munn 1998). Fish species with consistent and pronounced AChE activities may represent more sensitive sentinel species (Oliveira et al. 2007). In addition, it is important to understand the seasonal impact of agricultural effluents that affect the physiology of the resident fish species. Previous studies reported that *Rasbora caverii*, an indigenous Sri Lankan fish species, was more sensitive to AChE inhibition compared with the exotic Mozambique tilapia *Oreochromis mossambicus* (Wijeyaratne and Pathiratne 2006).

We have recently shown that *Garra ceylonensis*, *Devario malabaricus* and *Rasbora daniconius* (Teleostei, family: Cyprinidae) are quite widely distributed in agricultural areas in Sri Lanka (Sumith et al. 2011; Chapter 3). We designed field studies to examine the health of several species (Sumith and Munkittrick 2011a; Chapter 2). The aims of the present study were to understand the potential impacts of agricultural pesticides on native fish species by analyzing cholinergic effects on selected fish tissues and to investigate the possible correlation with known concentrations of AChE-inhibitory pesticides. The present study was conducted in the Uma-oya catchment in the upper Mahaweli River basin in Sri Lanka. We restricted our focus to OP and NMC insecticides during the assessment.

5.3 Materials and Methods

5.3.1 Study area

The Uma-oya catchment is the largest in the upper Mahaweli River basin in Sri Lanka, with a mean elevation of 1,678 (range 915–2,440 m) above mean sea level (MSL). Welimada (6°54'96"N 80°55'22"E) is one of the main townships within the catchment, with a total population of 107,369 (within the administrative division). The total discharge of the Uma-oya measured at the gauging station at Welimada was 23.4 million m^3 during the *Maha* monsoon season (October 2008 to March 2009) and 13.4 million m^3 in the *Yala* monsoon season (April to September, 2008) (Mahaweli Authority, Sri Lanka). Study sites were selected from the Uma-oya (stream) and tributary stream areas where agricultural and pesticide use patterns were known. The three study stream sections were situated in agricultural villages (village 1 = Medawela, 6°56'34"N 80°50'25"E, 1,110 m above MSL; village 2 = Girambe, 6°54'50"N 80°53'13"E, 1,054 m above MSL; and village 3 = Erabadda, 6°53'00"N 80°52'38"E, 1,106 m above MSL) in Uva-Paranagama and Welimada Divisional Secretariat divisions in the middle catchment (Figure 5-1); the representative catchment size was approximately 135 km^2 and the total agricultural land extent was 8,816 ha (Asian Development Bank 1997). The total agricultural area in these three villages is approximately 253 hectares of uplands and 125 hectares of lowlands (Provincial Department of Agriculture, Sri Lanka). The total rainfall during the year 2009 in the Welimada area was 1,897 mm with the highest monthly average rainfall in December (791 mm), and the lowest in June (5.8 mm). The average maximum and minimum air temperatures recorded in the area were 25.6 and 16.5°C, respectively (Department of Meteorology, Sri Lanka). Annual crops, such as vegetables and potatoes, are major land-use components that occupy the mountain slopes (i.e. uplands), valley bottoms, and plateaus (i.e. lowlands). Soil erosion is another characteristic feature in the catchment area and is caused by water runoff due to deforestation, intensive

agricultural practices on fields with steep slopes (>35°), and poor handling of water resources (Gunathilake and Gopalakrishnan 1999; Mungai et al. 2004).

The Knuckles Conservation Forest streams (7°27'82"N 80°48'56"E) were chosen for collection of reference fish specimens (Figure 5-1). Intense human activities in most of the areas were curtailed after the declaration of the area as a Conservation Forest in May 2000 by the Government of Sri Lanka. The Knuckles is covered with montane forests and an associated buffer zone of approximately 350 km^2, which spans from 200 to 1,900 m above MSL (Pinard et al. 2010). The Knuckles streams are part of the Amban-ganga catchment, which receives 1,435 to 2,111 mm of rainfall annually (as measured in six stations within the catchment during 2008–2010). A maximum air temperature of 31.5°C and a minimum of 20.2°C were recorded during 2008 and 2009 (Department of Meteorology, Sri Lanka).

5.3.2 Sampling strategy and storage for pesticide analysis

The target pesticides of OP and NMC classes of insecticides were selected from a list of most used pesticides in the three study villages in the catchment (Sumith and Munkittrick 2011b). Field samples (water and sediment grab samples) were collected in four representative months in two different seasons: during October and December, 2010 (*Maha* season) and June and August 2011 (*Yala* season), in periods overlapping active cropping in the Uma-oya catchment. To assess spatial variation in terms of contamination by pesticides, subsurface water in the mainstream and field canal, runoff water and sediment samples were collected from the following three sites in the Uma-oya (Figure 5-1): Medawela (upstream of the mainstream), Girambe (downstream of the mainstream), and Erabadda (tributary stream) surrounded by agricultural fields. To assess the potential of runoff input from agricultural fields, samples were collected twice during major rainfall events in the *Maha* monsoon season. Sediment samples were collected using a stainless steel corer and transferred into polyethylene bags, and duplicate water samples (subsurface and runoff water) were collected in 1-l amber-coloured glass bottles

following standard practices (US EPA, 2000, 2002). All samples were transported on ice in rigid-form boxes. In the laboratory, the sediment samples were air-dried, homogenized, and sieved (2-mm mesh size). Water and sediment samples were stored in the dark at 4°C (maximum storage time, two weeks) until analysis.

5.3.3 Pesticide analysis

The priority of pesticides were the OPs (chlorpyriphos, diazinon, dimethoate, profenophos, and phenthoate) and the NMCs (carbofuran, carbosulfan, and carbaryl). Water (subsurface and runoff in the mainstream and field canal) and sediment samples were analyzed for the presence of pesticides in the Chemical and Microbiological Laboratory of the Industrial Technology Institute, Colombo, Sri Lanka. Quality assurance and quality control of all analyses were ensured by participating in quality checking under the Swedish Board for Accreditation and Conformity Assessment (1791: ISO/IEC 17025) and the Sri Lanka Accreditation Board (ISO/IEC 17025, TL004) accreditation systems. Two manuals (AOAC 1990; Thier and Zeumer 1987) were used to create a combination of test methods (previously validated) that were followed for all compounds, using a HP 5890 gas chromatograph fitted with an Agilent 7973 Auto Sampler with electron capture and nitrogen–phosphorus detectors. Quantification and confirmation analysis were performed using HP-5 (5% diphenyl- polysiloxane and 95% dimethyl-polysiloxane) and HP-1701 (14% cyanopropyl-phenyl methyl-polysiloxane) columns (30 m x 0.32 mm x 0.25-µm film thickness) for gas chromatography-mass spectrometry. The column was held initially at a temperature of 160°C for 1 min, then increased at 10°C min^{-1} to 200°C, and finally at 300°C for 15 min. Temperatures of the injector and detector were maintained at 250 and 325°C, respectively. Helium was used as a carrier gas at a flow rate of 2.0 ml min^{-1}. A linear relationship between concentration and peak area was obtained within the range of 0.5 to 2.0 ng μl^{-1} with correlation coefficients greater than 0.99. Method detection limits (MDL) were greater than 1.0 µg l^{-1} for OPs and 2.0 µg l^{-1} for NMCs in water, and 0.02 and 0.04

mg kg^{-1} in sediment, for OPs and NMCs, respectively.

5.3.4 Fish sampling and tissue preparation

A maximum of ten fish was collected for each of three fish species (*G. ceylonensis*, *D. malabaricus*, and *R. daniconius*) with cast-nets (10-mm stretched mesh size) and stored at –21°C until analysis. Only sexually mature fish were selected from mixed-sex populations in each site (Table 5-1). Gender neutrality (Gruber and Munn 1998; Galgani et al. 1992; Kirby et al. 2000; Flammarion et al. 2002; Kopecka et al. 2004; Cong et al. 2006; Pathiratne et al. 2008; Mdegela et al. 2010) and seasonal stability (Chuiko et al. 1997; Kirby et al. 2000; Flammarion et al. 2002) were assumed for basal AChE activity in fish species based on previous studies. Measurements were taken of fork-length (± 0.1 cm), total body weight (± 0.001 g), and brain weight (± 0.001 g) after thawing, and the eyes and dorsal muscle from the cephalic region were excised and weighed (± 0.001 g); all tissues were stored at –21°C until analysis. Thawed tissues were thoroughly homogenized in an Eppendorf homogenizer on ice with the help of a micropestle, and 1 ml of ice-cold 0.1 M phosphate buffer was added (pH 7.4 prepared by mixing mono- and dibasic sodium hydrogen phosphate; Sigma-Aldrich, Germany). Each tissue homogenate was adjusted to 20% volume (w/v) by adding appropriate volumes of 0.1 M phosphate buffer (pH 7.4). Then, 1 ml of the tissue homogenates was removed to Eppendorf tubes and centrifuged (Sanyo® Micro Centaur) at 10,000 rpm (=7.826 *g*) for 5 min. The supernatant was used for AChE and protein analysis.

5.3.5 Acetylcholinesterase determination

Acetylcholinesterase activity was measured spectrophotometrically by using a modification of the Ellman et al. (1961) assay as adapted to a microplate reader by Hemingway (1998). Acetylthiocholine iodide (Sigma-Aldrich, Germany) was used as the substrate, and the resulting thiocholine was reacted with the colour-developing agent 5'-dithiobis-2-nitrobenzoic

acid (DTNB); Sigma-Aldrich, Germany), to give 2-nitro-5-thiobenzoic acid (NTB), a yellow-coloured anionic product with maximum absorption at 405 nm. Briefly, two 25-µl replicates of the enzyme preparation were added to adjacent wells of a 96-well microplate followed serially by 145 µl of 1% (v/v) Triton X-100 buffer (in 0.1 M of phosphate buffer, pH 7.8), 10 µl of 0.01 M DTNB (in 0.1 M of phosphate buffer, pH 7.0), and 25 µl of 0.01 M acetylcholine iodide (in distilled water). Acetylcholinesterase activity was determined immediately for 5 min at 12-s intervals with a BioTek® ELx808 microplate reader, and AChE specific activity was calculated by a computer-assisted program using the following formula:

AChE specific activity = OD_{405}/ [P] x E, where;

OD_{405} = optical density from AChE assay

P = protein concentration in the well (mg ml^{-1})

E = extinction coefficient of the substrate (OD_{405} μmol^{-1} ml^{-1})

AChE specific activity in fish tissues was expressed as µmol min^{-1} mg^{-1} protein.

All procedures were carried out on ice to minimize loss of enzyme activity. All assays were corrected for nonenzymatic activity with the same mixtures, except for using 25 µl of distilled water instead of the enzyme preparation. Results were expressed as a percentage of AChE activity in the inhibited fraction compared with control (uninhibited) activity. Protein concentrations of the individual homogenates were measured by Bio-Rad protein determination (Bradford 1976).

5.3.6 Statistical analysis

All data were first tested for normality and homogeneity of variance, and then analyzed by one-way analysis of variance (ANOVA), followed by a Tukey test using $p < 0.05$ as significant. Data are expressed as means ± SE. The AChE inhibition levels were calculated based on the mean AChE activity in control fish, and are presented as a percentage (± SE). Percent (%) inhibitions were compared with empirical values of exposure and/or effect; ≥20% as

mild exposure situation (Sancho et al. 2000), >50% as survivable (Fulton and Key 2001; Golombieski et al. 2008), and ≥70% as life-threatening (Fulton and Key 2001; Golombieski et al. 2008). Calculations of basic descriptive statistics on primary data and statistical analyses were performed using Minitab®, Ver. 16 for Windows.

5.4 Results

5.4.1 Organophosphate and NMC contamination patterns

Only four (i.e. chlorpyriphos, diazinon, dimethoate and profenophos) of five target OP insecticides and one (i.e. carbofuran) of three NMC insecticides were detected in any of the samples of subsurface, runoff water, and surface sediment, irrespective of the season. In relation to seasonal effects, an increase in concentration of all the detected OP and NMC insecticides was found during the *Yala* season. The spatial variations of cumulative residue levels of OP and NMC insecticides in water and sediment matrices are shown in Figure 5-2 and Figure 5-3, respectively. Water samples from background stream flow (A samples) and in field streamlets draining into the main flow (B sample) contained only chlorpyriphos (< 1 $\mu g\ l^{-1}$) at some sites. On a few occasions, dimethoate, carbosulfan, profenophos, phenthoate, chlorpyriphos, and diazinon were detected between <1 $\mu g\ l^{-1}$, and 3 $\mu g\ l^{-1}$. A similar situation was also observed for stream runoff collected during two sampling efforts during the *Maha* rainfall events in October and December (2010): profenophos and chlorpyriphos were detected at 1 $\mu g\ l^{-1}$ at some sites. Chlorpyriphos and diazinon were detected in sediments at concentrations of 16.36 $mg\ kg^{-1}$ (dry wt) in the *Yala* season. The principle occurrence of other pesticides could not be determined because of concentrations less than the method detection limits (1.0 $\mu g\ l^{-1}$ for OPs and 2.0 $\mu g\ l^{-1}$ for NMCs).

5.4.2 Acetylcholinesterase activity in fish species

The mean (± SE) lengths of fish specimens were not significantly different (ANOVA, $p > 0.05$) between sites and were 12.0 (± 0.18, n = 78) (*G. ceylonensis*), 7.8 (± 0.13, n = 57) (*D. malabaricus*) and 8.3 (± 0.14, n = 38) (*R. daniconius*). However, a significant weight difference was found between *G. ceylonensis* individuals at some sites (Table 5-1).

The differential tissue AChE inhibitory patterns in fish species, both in field and in simulator tests under various exposure scenarios, are presented in Table 5-2. Brain AChE activity was higher in January than in July in *G. ceylonensis* at all three sites in the Uma-oya, whereas muscle and eye showed the same seasonal difference only at the Medawela site (Figure 5-4). Brain activity levels were lower in July by 15.6% in Medawela, 26.3% in Girambe, and 36.4% in Erabadda than reference fish in the Knuckles (Table 5-3). The brain AChE activity in January samples from the Girambe and Erabadda sites were not significantly different compared with reference fish in the Knuckles. However, a significant inhibition of brain AChE activity of 29.5%, was found in fish from the Medawela in January compared with the Knuckles fish. All Uma-oya sites showed lower brain activity levels compared with the Knuckles reference populations (July; n = 20, $p < 0.001$), by variable inhibition degrees of 40.1 to 46.4%.

Significant differences ($p < 0.001$) were found between tissue-specific AChE activities and the specific activity decreased in sequence of brain > muscle > eye in *G. ceylonensis*. In unexposed fish, specific activity of AChE in brain was 51% higher (twofold) than those in dorsal muscle (1.27 and 0.62 μmol $min^{-1}mg^{-1}$ protein, respectively). Muscle AChE activity at all sites in all months in the Uma-oya was significantly depressed by 37.2 to 72.8% compared with the Knuckles fish. In the Girambe site, muscle AChE activity was depressed by 44.8% between January (n = 10) and July (n = 9) fish samples; seasonal AChE activity in the muscles was not significantly different in the Medawela and Erabadda sites (Figure 5-4). The AChE activity in the eye at all sites in all seasons was significantly depressed ($p < 0.001$) by 42.8 to 75.9%,

except at the Medawela site in January (2011), which showed a depression that was smaller by 17.9%. No significant differences in AChE activities in eye tissues were found between January and July samples from the Uma-oya.

Rasbora daniconius was only available at Erabadda out of three sites in the Uma-oya. The AChE-specific activities in the control populations (n = 17) of *R. daniconius* tissues were observed in a sequence of decreasing sensitivity as muscle = brain > eye. The corresponding baseline AChE-specific activities (mean ± SE) were 0.76 ± 0.05, 0.67 ± 0.04 and 0.22 ± 0.02 μmol min^{-1} mg^{-1} protein, respectively. Although the specific AChE activity difference between the brain and the muscle was smaller (13%), and not significantly different (ANOVA, $p > 0.05$), eye activity was reduced by 67 to 71% ($p < 0.001$) compared with reference fish. No significant differences were found in the brain AChE in control fish and January fish from the Erabadda site (Figure 5-5), although 60% inhibition was observed in July ($p < 0.0001$). A highly significant inhibition of 40 to 56% was found in muscle AChE activity in *R. daniconius* from the Erabadda site in January and July (2011) ($p < 0.0001$). Although not significantly different ($p > 0.05$), the muscle AChE activity was 27% less in July compared with January samples. No differences were found in mean AChE activity in eye tissue from any of the populations in the Knuckles and Uma-oya sites ($p = 0.57$) (Figure 5-5).

Devario malabaricus was available at two sites in the Uma-oya (Medawela and Girambe). The activity of AChE in the control population (n = 10) of *D. malabaricus* in the Knuckles streams was highest in muscle > brain > eye (Figure 5-6). The dorsal muscle and brain AChE-specific activities in *D. malabaricus* were significantly different ($p < 0.001$) by 43% high (1.7–fold). Both brain and dorsal muscle specific activities had significant differences from the eye, which had the smallest activity (1.39, 0.80, and 0.24 μmol min^{-1} mg^{-1} protein, respectively). Except in the Girambe site in January, all other brain AChE activities were significantly different ($p < 0.001$) from the Knuckles individuals. The minimum and maximum inhibitions on

brain AChE between the Knuckles and the Uma-oya populations were within a range of 34.2 to 47.8%, respectively. No significant differences were found in brain AChE activity (11.5%) between January and July samples in the Medawela site, whereas it was inhibited by 45.9% ($p < 0.001$) between January and July samples in the Girambe site.

The muscle AChE activity of all fish in Medawela and Girambe sites in the Uma-oya in both seasons were significantly depressed by 28.2 to 64.6% compared with the Knuckles counterparts. A depression in muscle AChE activity was found between January and July in the Madawela (11.4%) and Girambe sites (50.7%); only the latter was highly significant ($p < 0.001$). No significant differences ($p > 0.05$) were seen in mean AChE activity in the eye in any of the populations in the Uma-oya sites compared with the Knuckles (Figure 5-4).

5.5 Discussion

5.5.1 Contamination pattern

Organophosphate and NMC pesticides were detected at all sites in water during the *Yala* season, and rarely and only in low concentrations during the *Maha* monsoon season. The *Yala* vegetable cultivation season (June–September) is relatively drier than the *Maha* season in the Uma-oya catchment, and pesticides are used more heavily during the cultivation of vegetables compared with rice paddy. The small-scale vegetable fields are exorbitantly fertilized (Dharmakeerthi et al. 2011), and large amounts of pesticides (>23 kg of active ingredients ha^{-1} $year^{-1}$) are used for pest and disease control (Sumith and Munkittrick 2011a). Chlorpyriphos, diazinon, and carbosulfan had the greatest amount of agricultural application in the catchment (Sumith and Munkittrick 2011b), and chlorpyriphos, diazinon, and carbofuran were the dominant pollutants found. Chlorpyriphos and diazinon were detected in sediments at concentrations up to 16.36 mg kg^{-1} (dry wt). The Uma-oya catchment is characterized by exceptionally high soil erosion associated with topography (i.e. sloping lands), cropping patterns

and high intensity monsoonal rains. The entry and occurrence of pesticides in the Uma-oya catchment has not been studied, but similar catchment studies in Sri Lanka (Menike et al. 2010) and elsewhere (Schulz 2001; Dabrowski et al. 2002) have shown that contamination of the mainstream originates from adjacent fields and inputs into tributaries mainly *via* surface runoff and to a lesser extent by spray drift (Schulz 2001).

Despite the shorter lifespan of OP and NMC pesticides in environmental matrices, concentrations up to 138 $\mu g\ l^{-1}$ of OPs were detected in the present study (total of chlorpyriphos and diazinon). Menike et al. (2008) reported rainfall-induced chlorpyriphos concentrations of 2.48 $\mu g\ l^{-1}$ in a year-round assessment of stream water irrespective of doses applied in a similar agricultural catchment. Vegetable cultivation in the *Yala* season depends principally on non-rainfed irrigation methods; the crops are irrigated with water taken from irrigation canals usually at 2–3-d intervals, and the tailwater can be tainted with pesticides that have been applied during the preceding days or weeks. The upper and lower catenae of the lowland fields are composed of sandy-clay-loam to sandy-loam agricultural soils (with low organic matter content, 1.0–2.3%), affecting soluble and adsorbed fractions of pesticides in receiving waters. Many factors affect the transport of pesticides into aquatic environments, such as the manner, amount, frequency, and timing of application; the method of irrigation; the chemical properties of the pesticide; soil properties; land slope; and proximity to flowing water. Johnson (2007) showed an analogous situation where the mere application rate would not be a good predictor of the detection frequency because of a multitude of contingencies that determine the fate of a pesticide after it is applied. He showed that some pesticides, such as the insecticide chlorpyriphos have been detected less often than expected, solely on the basis of their application rates. Conversely, some pesticides, such as the herbicide atrazine, have been detected more frequently than expected. One of the major determinants of pesticide mobility is K_{oc} where larger pesticide losses have been associated with pesticides having low K_{oc} values compared with pesticides having high K_{oc}

values. In other words, for pesticides with low K_{oc} values, a larger fraction of the applied pesticide was transported off the field and into waterways.

5.5.2 Acetylcholinesterase activities

Higher basal values of muscle AChE activities in *D. malabaricus* and *R. daniconius* were observed in reference fish, whereas *G. ceylonensis* had higher basal activity in the brain. Lower basal activity of AChE was observed in the eye tissue in all species tested. Acetylcholinesterase activity in various fish tissues (e.g. brain, muscle, liver, eyes) has been used to indicate exposure to OP and NMC pesticides (Fulton and Key 2001; Nunes 2011). The brain AChE activities measured in *R. daniconius* in the reference area are in the same order of magnitude as those reported by Wijeyaratne and Pathiratne (2006) from *R. caverii* (a congeneric species) in a rice-growing area in the wet zone of Sri Lanka, although these authors reported AChE activity per whole body weight.

Baslow and Nigrelli (1961) suggested that a correlation exists between the general physical activity of fishes and their levels of skeletal muscle AChE, with higher AChE activities present in active than sluggish species. *Rasbora daniconius* and *D. malabaricus* showed higher levels of muscle AChE, and both species are active swimmers in the water column (Pethiyagoda 1991; McClure et al. 2006). In contrast, the pronounced brain AChE activity in *G. ceylonensis* compared with muscle is in accordance with its relatively sedentary nature as a benthic fish adapted for living on stony stream bottoms (De Silva 1991; Pethiyagoda 1991).

5.5.3 Acetylcholinesterase inhibition

Acetylcholinesterase inhibition in fish brain did not exceed 75% in any of the sites in any of the fish species; the highest inhibitions were in the range of 34.2 to 60%. A 73% inhibition in muscle AChE activity in *G. ceylonensis* was associated with intense exposure months in the *Yala* season. More interestingly, AChE inhibition was greater than 70% in *G.*

ceylonensis eyes in both *Yala* (i.e. 76%) and *Maha* (i.e. 72.5%) seasons, indicating particular sensitivity of eye tissue to inhibitors.

The highest inhibition of AChE (up to 60% in brain and up to 56% in muscle AChE activity in *R. daniconius*) occurred during the low-flow month in July (*Yala* season), when pesticide residues were more frequently detected. In lake environments (where pollutant accumulation and storage can be more likely), muscle ChE activities in Nile tilapia were inhibited by 37 to 46% during the rainy periods in comparison with the low extent of inhibition (21–25%) during the dry periods (Pathiratne et al. 2010). At the downstream site (Girambe), fish species showed more inhibition of AChE activity (both brain and muscle) than at the upstream (Medawela) site in the main-stream of the Uma-oya. Galgani et al. (1992) demonstrated that AChE activity in the muscles of the North Sea dab (*Limanda limanda*) varied according to the contamination gradient and was higher in less polluted waters.

Inhibited AChE activity in the muscle was apparent in *D. malabaricus* without significant responses in brain activity under low exposure periods to OPs and NMCs. Muscle AChE activity is thought to provide a protective role against the brain AChE inhibition in mosquito fish by supplanting active inhibitors (e.g. chlorpyriphos) (Carr et al. 1997). Acetylcholinesterase inhibition in eye tissue was less dramatic in *D. malabaricus* and *R. daniconius.*

The pattern of AChE inhibition is apparently stronger in different tissues of *G. ceylonensis* living in the tributary site (Erabadda) than in mainstream sites (Medawela and Girambe) in the Uma-oya. The high AChE activity in "clean" environments and reduced activity in "polluted" areas is fairly typical (Galgani et al. 1992; Kirby et al. 2000; Podolska and Napierska 2006). However, more detectable pesticide residues were found in the tributary site than in mainstream sites, with no contrasting differences in cropping and pesticide use patterns in the three study sites (Sumith and Munkittrick 2011b). Despite high pesticide inputs into the

stream through associated field canals (as has been observed in the Erabadda site), the differences in total OP and NMC concentrations in two mainstream locations (4–24 μg l^{-1}) and the tributary location (20–40 μg l^{-1}) were not associated with different AChE inhibition. This is not surprising because similar AChE inhibition (86–92%) has been seen in fish brain under diazinon exposures over a range of 42 to 450 μg l^{-1} during 24–96-h exposure (Cerón et al. 1996; Pan and Dutta 1998; Gaworecki et al. 2009).

Acetylcholinesterase activity inhibition has been correlated with dominant AChE-inhibitory compounds (see also Forget et al. 2003; Takao et al. 2010). The narrow range of SE values (0.01–0.10) for specific AChE activity suggests a wider exposure to contaminants (i.e. diffuse nature of contaminants), as variability can be higher with localized point pollution (Kirby et al. 2000; Kopecka et al. 2004). Other AChE-modulating compounds were present that were not detected frequently. For example, carbofuran, a strong AChE inhibitor, was detected in one of the three sites in the Uma-oya (i.e. a tributary drainage at Erabadda) at a concentration up to 16 μg l^{-1}, which has the potential to inhibit fish brain AChE up to 59 to 80% at 10 to 50 μg l^{-1} (Bertrand et al. 1998; Chandrasekara and Pathiratne 2007) or even lower concentrations (Dembelé et al. 2000).

Inhibition of AChE activity has mainly been reported in studies performed with pesticides (Salbego et al. 2010; Mdegela et al. 2010), but different effects have also been seen with metals on fish brain (Rita et al. 2003; Sant'Anna et al. 2011) and muscle AChE activity (Rita et al. 2003), including cadmium (Silva and Pathiratne 2008; Pretto et al. 2010), mercury, and lead (Richetti et al. 2011). Miron et al. (2005) reported increased AChE activity in fish tissues by some contemporary classes of herbicides such as quinclorac and metsulfuron-methyl. Therefore, AChE inhibition as an effective biomarker of pesticides in real-exposure situations, such as in a diffused agricultural exposure scenario, is rather limited (Almeida et al. 2010; Adedeji 2011) unless it is known that no other anti-cholinesterase agents are present in the

system at concentrations that may cause AChE inhibition. Earlier studies in the Uma-oya stream and tributary streams reported excess Pb and Cd concentrations in water and sediment close to agricultural and urban areas (Ranasinghe et al. 2008; Bandara et al. 2011).

Wijeyaratne and Pathiratne (2006) showed *R. caverii* to be markedly more sensitive to brain AChE depression compared with exotic tilapia (*O. mossambicus*), suggesting that *Rasbora* may be more sensitive than exotic species. Many organisms produce different cholinesterasic forms in their muscle tissue (Kozlovskaya et al. 1993), and tilapia have multiple forms (Rodríguez-Fuentes and Gold-Bouchot 2004), but, cyprinid fishes have shown AChE activity only in their muscles (Lundin 1962). *Danio rerio*, which is one of the closely related lineages to *D. malabaricus*, is confirmed to have only one functional AChE gene (Bertrand et al. 2001). Thus the heightened tolerance of exotic tilapian species (Fulton and Key 2001) may be due to their diverse forms of cholinesterase activities.

The less dramatic AChE inhibition in the eye tissues in *D. malabaricus* and *R. daniconius* in both seasons indicates another example of the protective capacity of muscle AChE in fish. The existence of no AChE inhibition in the eye tissues despite mild (39.9% in the Erabadda site) or higher (54.1% in the Medawela site) inhibitory effects on muscle AChE activity is consistent with the above observation. Many planktivorous fish are visual foragers, highly dependent on light to efficiently detect and consume their zooplankton prey. Therefore, it can be argued that the protective capacity of muscle AChE for the brain (as discussed above) can be extended to other tissues that have critical importance for survival. The visually-aided feeding habit of *R. daniconius* has been confirmed by Pet and Piet (1993) by their preference to concentrate on the surface and in littoral zones in open waters. However, *G. ceylonensis* behaved dissimilarly; this fish species is a bottom-dwelling algae-eater (benthivorous), for which intense visual activity may not be advantageous. Therefore, it can be suggested that inherently low

"buffering capacity" of fish tissues can be exploited as sensitive AChE assessment targets in environmental monitoring.

5.5.4 Possible implications for fish survival

The range of AChE inhibitions seen was generally <60% (Table 5-3). A 20% or greater inhibition has been shown to indicate a mild exposure situation (Morgan et al. 1990; Sancho et al. 2000), and life-threatening exposures are thought to occur at greater than 70% (Fulton and Key 2001; Golombieski et al. 2008). Fulton and Key (2001) reported instances of survivability of some fish species under intense inhibition in excess of 95% in brain AChE. Chandrasekara and Pathiratne (2007) reported that the maximum inhibition of AChE in brain tissues at which mortality of *O. niloticus* occurred was 85% for chlorpyriphos (i.e. an OP insecticide) and 64% for carbosulfan (i.e. a NMC insecticide). Several studies have shown less correlation between muscle cholinesterase activity and swimming performance and/or velocity in fish exposed to fenitrothion (an OP insecticide) and carbofuran (a NMC insecticide) (Almeida et al. 2010; Hernández-Moreno et al. 2011).

The severity of inhibition can depend on the compound and the fish species. Carr et al. (1997) showed that brain AChE was inhibited up to 87 to 93% in largemouth bass (*Micropterus salmoides*), bluegill sunfish (*Lepomis macrochirus*), and golden shiners (*Notemigonus crysoleucas*), whereas only 73% inhibition was found in the mosquitofish (*Gambusia affinis*), with no lethality after exposure to the same concentrations of chlorpyriphos. Recently, a whole-effluent environmental risk assessment approach in agricultural catchments has highlighted the delineation of complex-mixture ecotoxicological hazards to aquatic organisms (Martins et al. 2007; Ansara-Ross et al. 2009). The recent investigations (Chapter 4; Sumith and Munkittrick 2011c) revealed that in sites receiving high agricultural inputs (i.e. the middle catchment of the Uma-oya), sub-optimum gonadal development and fecundity were present in Ceylon stone sucker (*G. ceylonensis*) populations, suggesting a gross impairment of fish physiology. This

particular effects-based assessment approach is based on the premise that holistic adverse effects in fish expressed as "effect" can be observed at lower levels of biological organization (Munkittrick et al. 2000; Dubé 2003). One key challenge to understanding this relationship is linking pesticide effects in individual fish to the intrinsic productivity of populations (see Chapter 1).

It has been shown that AChE inhibition predisposes fish to impacts on a number of physiological processes. Physiological stress is an earlier form of an effect and over stress can lead to death of organisms. Acetylcholinesterase enzyme activity is important in this respect, because acute and chronic phases are distinguishable depending on the inhibitor concentration. Sandahl et al. (2005) began to address this relationship by showing that exposures to low, environmentally realistic concentrations of OPs (e.g. chlorpyriphos) have implications on several bioenergetic functions. Acetylcholinesterase inhibition in fish can result in loss of equilibrium, decreased respiratory effectiveness, altered swimming performance, and reduced food consumption (Wijeyaratne and Pathiratne 2008; Gaworecki et al. 2009), the most commonly reported effects of OP and NMC exposure. The reductions in feeding are likely to lead to reductions in the size of gonads and fecundity, an end point that has been shown to be an important determinant of ecological competence and survival in individual fish (Dutta and Maxwell 2003). Dutta and Maxwell (2003) showed that a sub-lethal dose of 60 $\mu g\ l^{-1}$ of diazinon changed the microscopic structure of ovaries in bluegill (*Lepomis macrochirus*) including destruction of follicles, increased intrafollicular spaces, vacuolated cytoplasm, increase of atretic follicles, and shrinkage, leading to the production of fewer viable eggs and affecting the population dynamics of species in polluted environments.

Up to 85% inhibition of muscle AChE activity has been observed in *O. niloticus* during a 96-h exposure to sub-lethal doses of trichlorfon (an OP insecticide) (Guimarães et al. 2007). The toxic effects from continuous exposure (i.e. *in vitro* experiments) are not always adaptable

to intermittent exposure events occurring in the field (Sumith et al. 2009). At present, findings are conflicting as to whether the condition factor (K) of fish species would reflect pesticide impact under *in situ* conditions. In previous field studies in streams adjacent to rice fields, Wijeyaratne and Pathiratne (2006) observed that the condition factor of *Rasbora caverii* could not be interpreted as an effect of pesticides and concurrent AChE inhibition.

An intense inhibition of AChE activity in the eyes of *G. ceylonensis* up to 76% can be potentially severe enough to disrupt the overall survivability of fish in their natural environments, if not life-threatening. In polluted environments, the suppression of eye AChE activity in fish might lead to impaired optomotor response (i.e. the ocular reaction to movement or light resulting in body motion either toward or away from the stimulus). Pan and Dutta (1998) observed limited optomotor reactions in largemouth bass, *Micropterus salmoides* that is disrupted food-searching, orientation toward food odour, searching for mates, and locating and avoiding predators after exposure to sub-lethal concentrations of diazinon. Sahib et al. (1980) stated that differential inhibition of AChE activity in fish tissues (i.e. brain, muscle, eyes) may be due to the presence of isozymes with different affinities for the substrate and the inhibitor. They also stated that the inhibitory pesticides can be present in different amounts in the different tissues, producing differential inhibition, or the inhibitor may be metabolized at different rates.

Insignificant effects on condition factor (K) in fish species exposed to cumulative agricultural stressors (Chapter 4) would indicate changes in energy storage, metabolism and feeding activity, may have encompassed physiological tolerance levels. It is considered that the initial stress response is adaptive, and a fish can overcome the disturbance, and regain its normal or homeostatic state. If the stressor is severe or long-lasting, however, the fish may no longer be able to cope with it and, as a result, enters a maladaptive or distressed state leading to decreased performance, a pathological condition or possibly death (Bond 1996).

In conclusion, evidence from the present study confirms that AChE inhibition is useful as an exposure biomarker for the presence of neurotoxic contaminants in the agricultural catchments of Sri Lanka. Exposure to AChE-inhibitory OP and NMC insecticides is shown to be higher in the *Yala* season than in the *Maha* season in the Uma-oya catchment. Inhibition in fish species showed the same concentration patterns of AChE-inhibitory OP and NMC insecticides across seasons. In the present study, because control fish samples were caught in the *Yala* season, and no significant differences were found in stream temperature, air temperature and rainfall profiles between catchments (Chapter 4; Sumith and Munkittrick 2011a), the seasonal influence on baseline AChE activity was ignored. However, divergent opinion in the literature exists as to whether temperature influences AChE activity in fish (Chuiko et al. 1997; Kirby et al. 2000; Flammarion et al. 2002; Durieux et al. 2011), thus preventing extensive generalizations. Even though it is impossible to correlate the findings of AChE inhibition with the presence of exclusive OP and NMC insecticides, the results suggest that other contaminants can act synergistically, cumulatively, or antagonistically in complex mixtures contributing to the overall impact on AChE activity. However, determining AChE activity and using this biomarker as an early warning signal of exposure and/or adverse effects can still be effective in the future to study spatial and temporal trends in the quality of waters running in agricultural catchments.

Our findings, along with previous observations of soil transport into the stream, have implications for planning irrigation and soil conservation measures in the Uma-oya catchment in terms of aquatic life conservation. If contaminant discharge is a serious problem during the *Yala* cultivation season, management options could focus on specific issues occurring in the system. Depending on the stream section, where active land preparation practices prevail, flashy currents of soil-laden water could also occur, and these would transport pesticides off the vegetable fields. Christen et al. (2005) noted some regulatory options instituted in Australia to curtail pesticide movement from the farm field that is by practicing a "withholding period" after

pesticide application during which water is not released from the fields, and constructing high banks to ensure rainfall is retained within the field. However, this is not practically feasible in a location like the Uma-oya catchment. This is primarily a question of farm field size and soil/geological characteristics. Stringent pesticide management options and good agricultural practices would be required to protect fish species in agricultural catchments.

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Table 5-1. Descriptive data on fork-length (L) and total weight (W) of fish species used for AChE analysis.[a]

Species	Site	N	L ± SE	L (min)	L (max)	W ± SE	W (min)	W (max)	p-value
GC	ME	20	13.36 ± 0.36	9.5	15.2	26.84 ± 2.40	12.24	46.15	0.126 (L)
	GI	19	12.58 ± 0.32	9.6	14.5	29.28 ± 1.96	12.89	43.26	<0.001 (W)
	ER	19	12.07 ± 0.35	9.8	15.5	26.14 ± 2.50	11.69	55.86	
	RO	10	12.09 ± 0.59	9.5	15.0	43.53 ± 6.79	12.38	77.20[b]	
	IK	10	11.01 ± 0.41	9.3	12.4	19.81 ± 2.05	10.97	27.85	
DM	ME	18	7.43 ± 0.16	6.4	8.5	5.86 ± 0.41	3.62	8.72	0.290 (L)
	GI	19	7.97 ± 0.31	6.5	10.9	7.22 ± 0.86	3.32	16.27	0.302 (W)
	RO	10	7.98 ± 0.17	7.2	8.7	6.88 ± 0.50	4.63	9.87	
	IK	10	7.92 ± 0.20	7.0	8.8	5.76 ± 0.41	4.12	8.24	
RD	ER	19	8.33 ± 0.21	6.7	10.2	8.19 ± 0.59	4.16	13.7	0.435 (L)
	RO	10	8.01 ± 0.26	6.5	9.7	6.40 ± 0.69	3.58	11.67	0.137 (W)
	RM	9	8.52 ± 0.29	7.4	9.6	8.25 ± 0.74	5.03	11.61	

GC = *G. ceylonensis*, DM = *D. malabaricus*, RD = *R. daniconius*

N = Number of fish.

For descriptive site codes, see Figure 2-1 legend. RO = Rambukoluwa , IK = Illukkumbura and RM = Ranamuregama are reference sites in the Knuckles conservation forest area.

[a] Within a column, for each species, mean L and W among sampling sites are not significantly different (ANOVA, Tukey test $p > 0.05$).

[b] Significant difference from rest of the sites for *Garra ceylonensis*.

Table 5-2. Comparison of AChE inhibitory levels in various fish tissues on pesticide exposure reported in the literature.

Fish species	Tissue	Target pesticide	Concentration	Exposure situation	Inhibitory level (%)	Reference
Nile tilapia	Brain	Carbofuran	10 µg/L	Static, non-renewal (48-h)	59	Chandrasekara and Pathiratne 2007
European eel *Anguilla anguilla*	Brain	Diazinon	42 µg/L	6-h	47	Cerón et al. 1996
				72-h	89	
Hybrid striped bass (*Morone saxatilis* × *M. chrysops*)	Brain	Diazinon	102 µg/L	72-h	86.4	Gaworecki et al. 2009
Largemouth bass *Micropterus salmoides*	Brain	Diazinon	450 µg/L	Static, non-renewal (24-h)	91.5	Pan and Dutta 1998
Common carp *Cyprinus carpio* L.		Carbofuran	0.22 µg/L	-	75	Dembelé et al. 2000
Common carp *Cyprinus carpio* L.	Brain	Carbofuran	0.05 mg/L	48-h	80	Bertrand et al. 1998
Goldfish *Carassius auratus*	Brain	Carbofuran	5 µg/L	Static, non-renewal (12-h)	8.6	Bretaud et al. 2000
	Muscle		50 µg/L	do – (48-h)	23	
	Brain		500 µg/L	do – (48-h)	87	
	Muscle		500 µg/L	do – (48-h)	93	
Zebrafish *Danio rerio*	Whole fish	Parathion	0.9 µg/L	-	73	Roex et al. 2003
Eel *Anguilla anguilla*	Brain	Fenitrothion	0.02 mg/L	-	40	Sancho et al. 1997
Eel *Anguilla anguilla*	Eye	Thiobencarb	-	Flow through	75	Sancho et al. 2000
European sea bass *Dicentrarchus labrax* L.	Brain	Fenitrothion	0.06 mg/L	Static, renewal	26	Almeida et al. 2010
European sea bass *Dicentrarchus labrax* L.	Muscle	Fenitrothion	2 mg/L	Static, renewal	69	Almeida et al. 2010
Rasbora caverii	Brain	Fenthion	3 µg/L	Static, non-renewal	54	Wijeyaratne and Pathiratne 2006
R. caverii	Brain	Phenthoate	5 µg/L	Static, non-renewal	77	Wijeyaratne and Pathiratne 2006

Table 5-3. *In vivo* inhibition of AChE activities in brain, muscle and eyes homogenates from *Garra ceylonensis*, *Devario malabaricus*, and *Rasbora daniconius* in relation to the background aggregate exposure concentrations of OP and NMC insecticides in the Uma-oya in the *Maha* (2010) *Yala* (2011) seasons.

Site	Season	Aggregate residue concentration ($\mu g\ l^{-1}$)[a]	Fish species	Brain (% inhibition)[b]	Muscle (% inhibition)[b]	Eye (% inhibition)[b]
ME	*Yala*	38	GC	40.1	58.3	42.8
			DM	41.8	59.3	27.5
	Maha	ND[c]	GC	29.5	44.6	17.9
			DM	34.2	54.1	NIL
GI	*Yala*	46	GC	43.9	65.4	65.8
			DM	47.8	64.6	7.7
	Maha	6	GC	23.9	37.2	46.4
			DM	3.7	28.2	NIL
ER	*Yala*	213	GC	46.4	72.8	75.9
			RD	59.9	56.1	15.9
	Maha	ND[c]	GC	15.8	52.5	72.5
			RD	4.4	39.9	NIL

GC = *G. ceylonensis*, DM = *D. malabaricus*, RD = *R. daniconius*
ND = Not detected
NIL = No inhibitory levels
For descriptive site codes, see Figure 5-1 legend.
[a] Aggregate OP and NMC insecticides measured in the water samples.
[b] Each value represents percentage of AChE activity in the inhibited fraction compared to the control (uninhibited) activity.
c Pesticide concentrations were below the method detection limit, 1.0–2.0 $\mu g\ l^{-1}$.

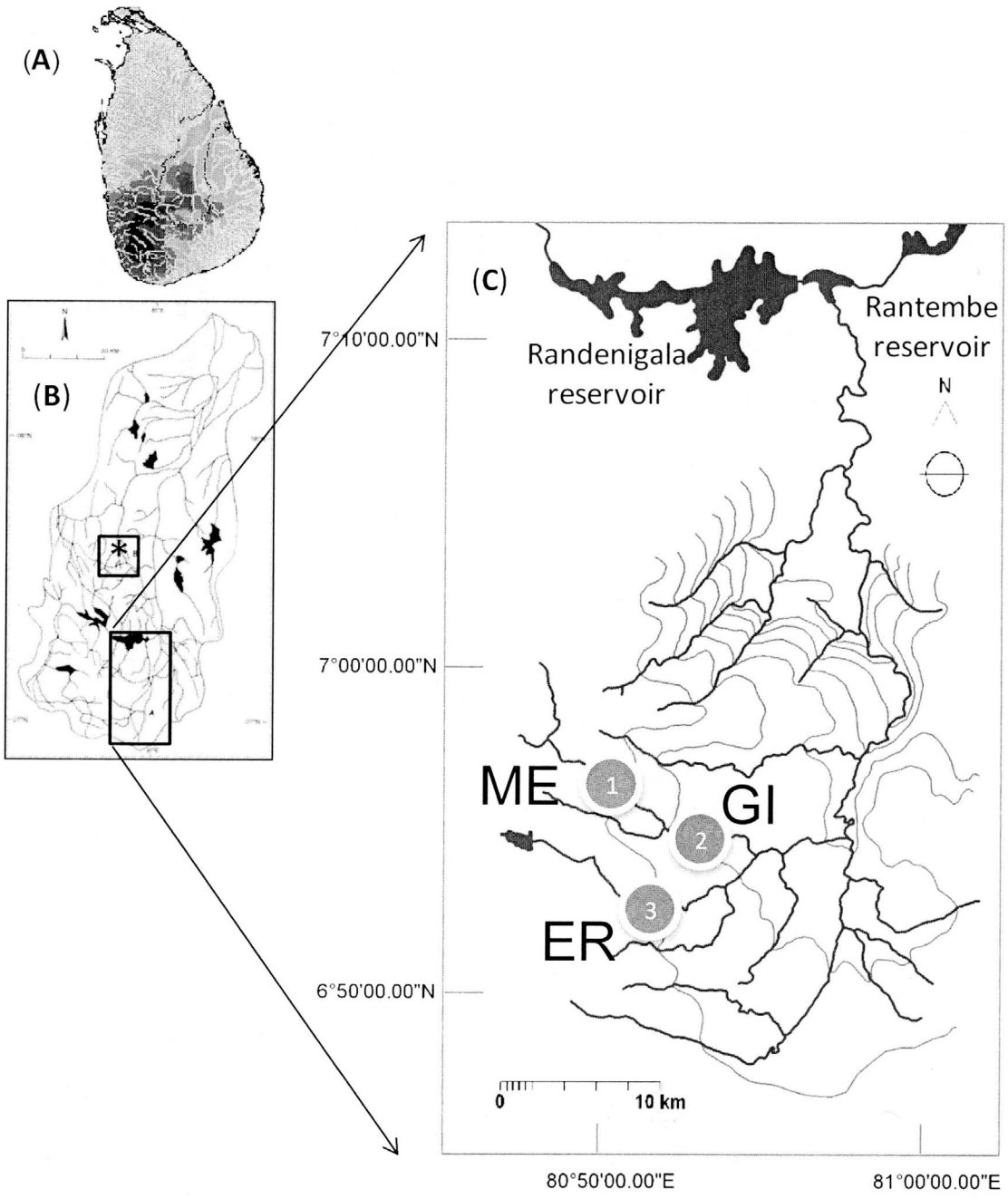

Figure 5-1. Locations of three sampling stations for collection of fish, subsurface, and runoff water and sediment in the Uma-oya catchment (upper Mahaweli River tributary, Sri Lanka). Asterisk indicates the location of the Knuckles streams for reference fish collection. (A) Map of Sri Lanka. (B) Map of stream distribution of Mahaweli River basin. (C) Map of stream distribution of the Uma-oya. ME = Medawela, GI = Girambe, ER = Erabadda.

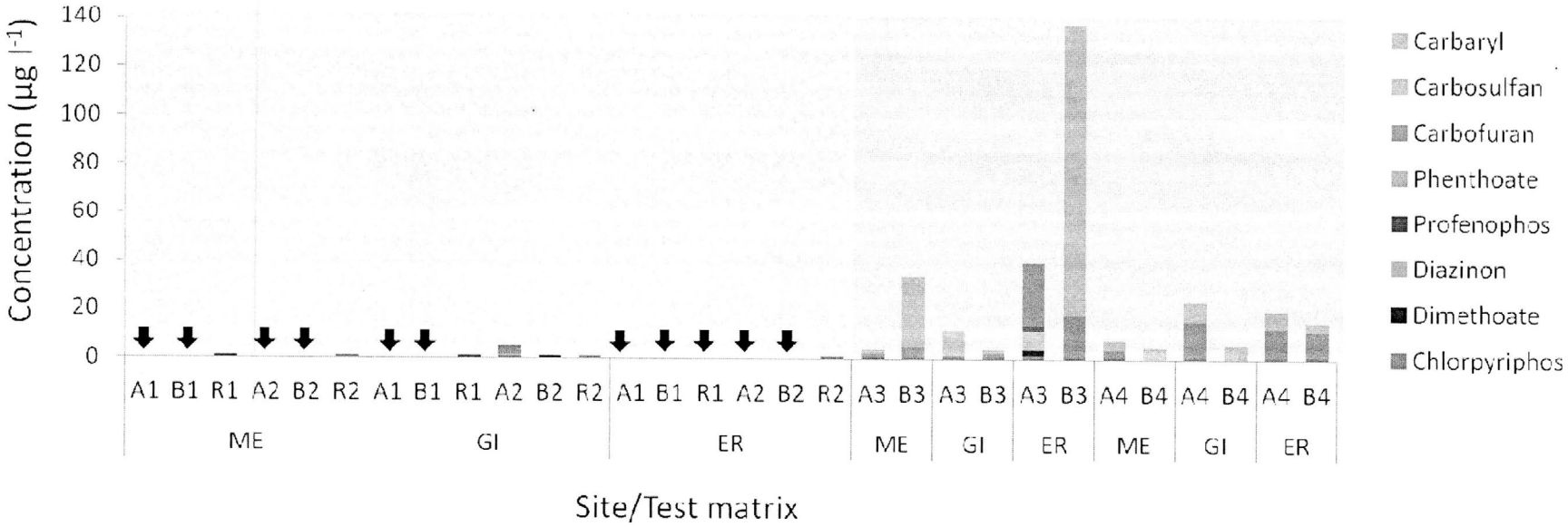

Figure 5-2. Organophosphate (OP) and N-methyl carbamate (NMC) insecticide concentrations in the water samples collected in the Uma-oya catchment. Arrows denote sampling occasions on which pesticide concentrations were below the detection limit of the method (1.0–2.0 µg l^{-1}). Shaded areas indicate *Maha* (yellow area left) and *Yala* (blue area right) sampling seasons. Typically, the *Maha* season is October to January. Samplings were conducted in October (A1, B1, and R1) and December (A2, B2, and R2) in 2010. The typical *Yala* season is April to July. Samplings were conducted in June (A3 and B3) and August (A4 and B4) in 2011; A1 to A4 = main-stream subsurface water flow; B1 to B4 = field canal to main flow; R1 and R2 = main-stream runoff. For descriptive site codes, see Figure 5-1 legend.

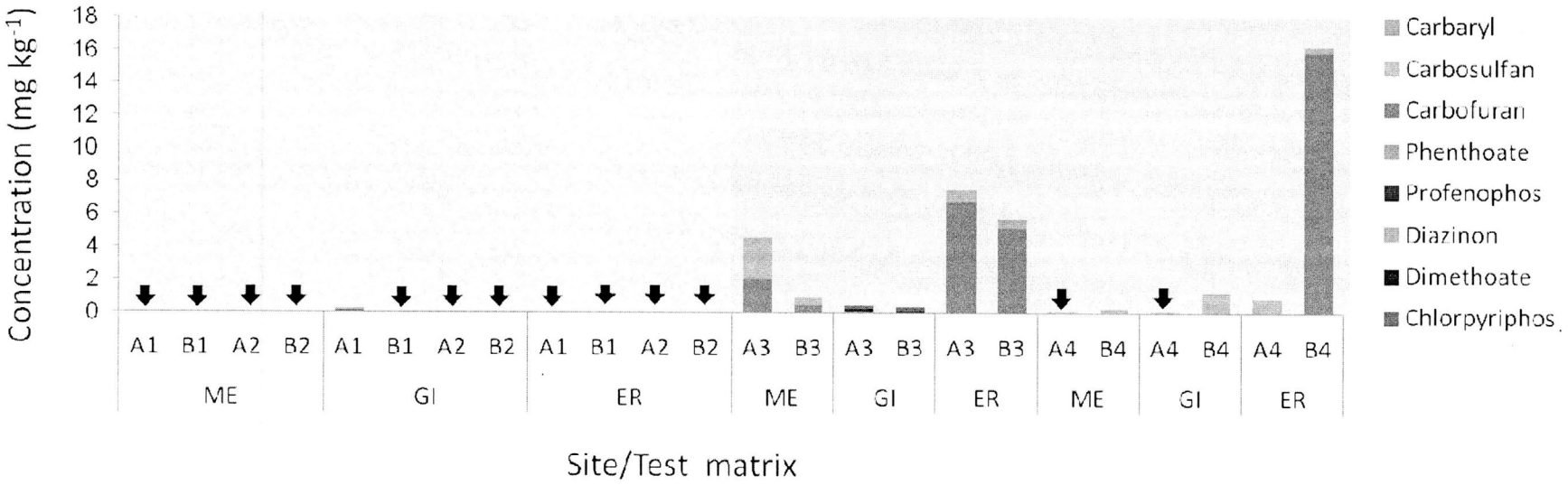

Figure 5-3. Organophosphate (OP) and N-methyl carbamate (NMC) insecticide concentrations in the sediment samples collected in the Uma-oya catchment. Arrows denote sampling occasions on which pesticide concentrations were below the detection limit of the method (0.02–0.04 mg kg^{-1}). Shaded areas indicate *Maha* (yellow area left) and *Yala* (blue area right) sampling seasons. Typically, the *Maha* season is October to January. Samplings were conducted in October (A1 and B1) and December (A2 and B2) in 2010. The typical *Yala* season is April to July. Samplings were conducted in June (A3 and B3) and August (A4 and B4) in 2011; A1 to A4 = bed sediment in the main-stream flow; B1 to B4 = bed sediment in the field canal to main flow. For descriptive site codes, see Figure 5-1 legend.

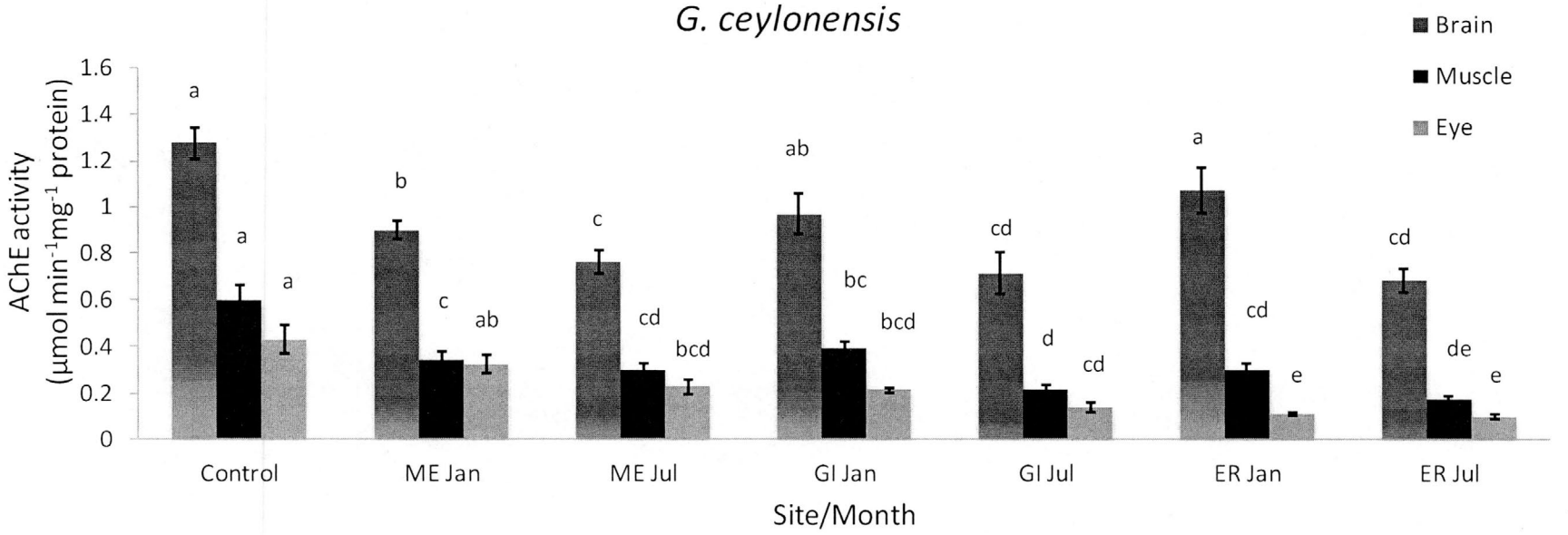

Figure 5-4. Seasonal pattern of AChE activity in brain, muscle and eye tissues in *Garra ceylonensis* in the Uma-oya in comparison with the Knuckles counterparts. Values are mean ± SE. Means of each tissue that do not share a letter are significantly different (ANOVA, Tukey $p < 0.05$) compared with the control. For descriptive site codes, see Figure 5-1 legend. Control fish were sampled in July, 2011 (corresponding to *Yala* season).

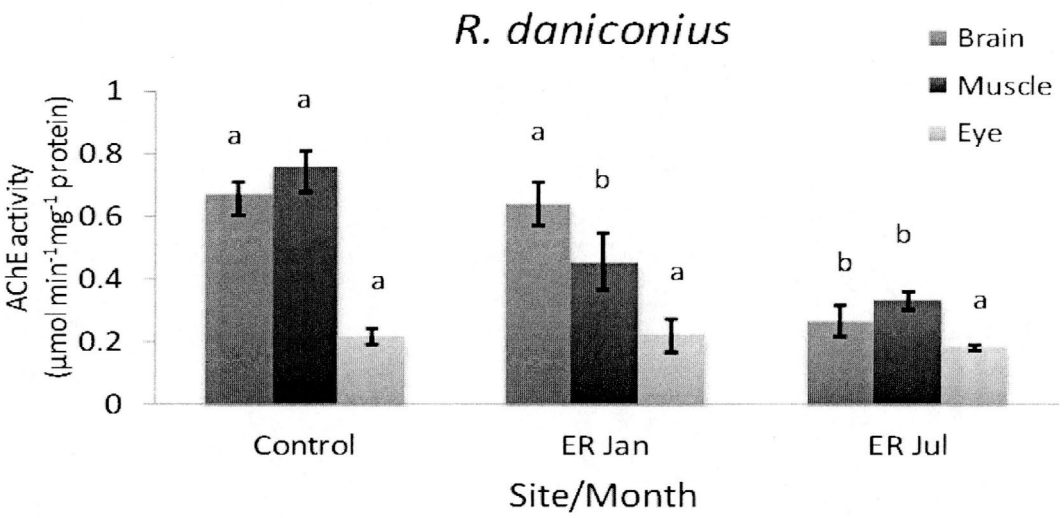

Figure 5-5. Seasonal pattern of AChE activity in brain, muscle and eye tissues in *Rasbora daniconius* in the Uma-oya in comparison with the Knuckles counterparts. Values are mean ± SE. Means of each tissue that do not share a letter are significantly different (ANOVA, Tukey $p < 0.05$) compared with the control. For descriptive site code, see Figure 5-1 legend. Control fish were sampled in July, 2011 (corresponding to *Yala* season).

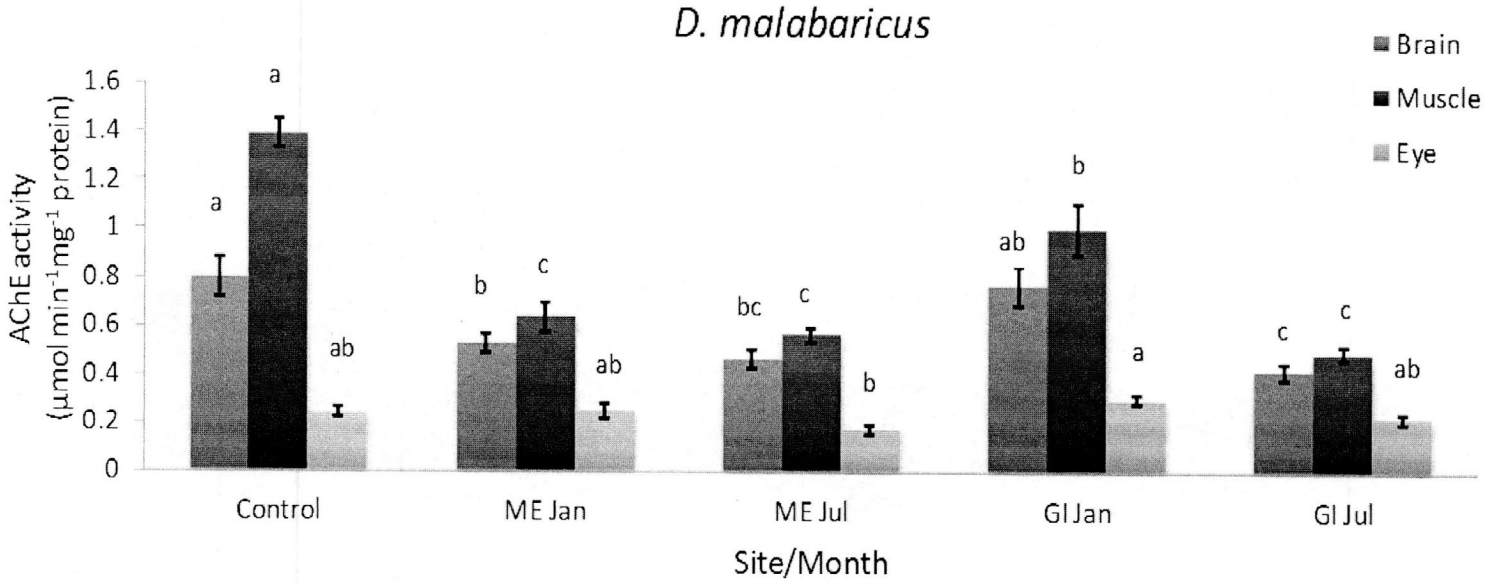

Figure 5-6. Seasonal pattern of AChE activity in brain, muscle and eye tissues in *Devario malabaricus* in the Uma-oya in comparison with the Knuckles counterparts. Values are mean ± SE. Means of each tissue that do not share a letter are significantly different (ANOVA, Tukey $p < 0.05$) compared with the control. For descriptive site codes, see Figure 5-1 legend. Control fish were sampled in July, 2011 (corresponding to *Yala* season).

CHAPTER 6

6 General Discussion

Agriculture has intensified on a global basis as a result of the increasing demands for food production for ever-growing human populations. November, 2011 was remarkable for the birth of 7th billion human on the planet and the Asian region represents the most densely populated area. The increasing population base and scarcity of resources (i.e. land, water, etc.) will mean further intensification of crop production and crop protection inputs (i.e. fertilizer, pesticides) at the expense of environmental health. Environmental preservation goals will depend on how intensified agricultural systems are managed in response to other concurrent pressures. Agricultural inputs are essentially non-point sources that are diffused over space (Corwin and Wagenet 1996) making them more difficult to quantify. At the same time, agro-ecosystem studies are becoming increasingly popular as metaphors for "near-natural" systems (Roger et al. 1994).

Ancient human civilizations originated in association with the great river systems of the Euphrates, Tigris, Nile and Indus, and the present-day human settlements and activities are constantly in association with water bodies. The contamination of adjacent water bodies is inevitable. Assessing the ecosystem health of running rivers is often complicated because these ecosystems receive multiple, interacting effluent discharges. Confounding factors, such as complex mixing hydraulics and historical loading effects, can result in equivocal field data that provide weak inputs for ecological risk assessments (Cash et al. 2003). In addition, limited knowledge of the concentration and duration of stressor exposure, non-independence of treatments, lack of appropriate replication and excessive spatial heterogeneity can complicate the identification of causality (Culp et al. 1996, 2000).

The Canadian Environmental Effects Monitoring (EEM) assessment methodology has been practiced for more than two decades (Dubé et al. 2002), and various monitoring methods have been proposed to better understand the impacts of contaminants and responses of organisms (or *vice versa*) such as those in artificial streams (Culp et al. 1996; Cash et al. 2003) and in-stream enclosures (Courtenay et al. 1998). None of these approaches have been perfected to meet the requirements of regulatory agencies (Shaw and Kennedy 1996), but there are a wide variety of methods becoming available (Crane et al. 2007).

Assessing the relationship between agricultural stressors and fish reproductive development has been a primary target for population impact assessments. Fish reproduction is essential to the survival of the species, and is highly sensitive to environmental disturbance. In general, agricultural field runoff includes pesticides (Schulz et al. 1998; Neumann and Dudgeon 2002), and sediments (Schulz 2001) and both may degrade the water quality transiently after heavy rainfall events (Ntow et al. 2008). However, runoff and sediment constituents, such as particulate matter (with more or less organic composition) and soil particles have shown to have a buffering effect on pesticide impacts on aquatic organisms (Carriquiriborde et al. 2007). Fish are likely to be affected by other agricultural activities through secondary introduction of pesticides to watercourses by cleaning and discarding equipment rinsate carelessly, or by spraymen using local water bodies for washing equipment (see Sumith and Munkittrick 2011).

Cumulative Effects Assessment (CEA) has been defined as the process of evaluating the collective impacts posed by individual actions in a manner that is additive, synergistic and antagonistic on the environment that result from past, present and foreseeable future actions (Seitz et al. 2011). Agricultural inputs represent an incidence of a potential cumulative impact from the multiple stressors. To assess significance in the context of cumulative impacts, the important resources, actions, and their cause-and-effect relationships have to be well characterized.

This dissertation is taking the challenge of evaluating reproductive stress in a geographic setting (i.e. south and southeast Asia) completely unexplored previously for cumulative impact and multiple stressor assessment on fish populations in agricultural areas. The main goal of my dissertation is the application of an stressor- and effects-based iterative assessment approach to better understand the cumulative agricultural impact on resident fish species in Sri Lankan streams. When I started the research, there were significant challenges for conducting this kind of an assessment in Sri Lanka, including that the fish community in Sri Lanka was poorly identified, potential sentinel species were unknown, exposures and residency timing for potential sentinels was unknown, the sample timing and target samples sizes were unknown, reference sites were unknown, and the acceptable study design was unknown.

6.1 Summary of dissertation

The critical requirement in this activity is to define the baseline condition of the resources and the ecosystem beyond which adverse or beneficial change would cause significant degradation or enhancement of the resources, respectively. The assessment of ecological significance is then made by comparison to the baseline condition. In the context of most agricultural catchments, this baseline condition has not been studied and/or defined, although such systems may have been stressed and have conspicuously changed over the years or for decades. Alternatively, fish populations may have been exposed to contaminant stressors over many generations and may have acquired some level of adaptive response or reduced sensitivity to these stressors. Such adaptive responses to xenobiotics (e.g. pesticides) are well known in some species taxa (see review by Clements and Rohr 2009).

In order to achieve my goal of developing an acceptable study design, I examined several hierarchical steps of environmental assessment by known and experimental biology of most abundant three fish species. It looked at life history characteristics and suitability for

providing information to acquire specific cumulative effects in afflicted environments. The specific challenges in intensive agricultural catchments are selecting study sites and reference data (Chapter 2), potential sentinels within a fish community (Chapter 3), responses in fish populations (Chapter 4) and an environmentally-realistic biochemical tool (Chapter 5) for impact assessments. My dissertation contributes to these areas of study design approach by examining the potential synergies and complications in each of them, and in so doing sheds light on declaring components of impact. The next sections describe the specific findings of four chapters of my dissertation in sequence, and suggest some follow up studies for elucidating specific processes occurring in the system and mechanisms of impact.

The present study was conducted iteratively with adaptive sampling in the absence of well defined baseline data or assessment protocols for tropical streams. Before designing any impact study, it is important to determine exactly how contamination is occurring, as agricultural inputs are likely to be of seasonal and episodic importance as compared with sewage water inputs which would be more continuous. We conducted a preliminary survey of farmer attitudes, cropping and pesticide use patterns to set the sampling stages for this study (Sumith and Munkittrick 2011). The per unit area pesticide consumption (= total pesticide used/surveyed area) revealed that the environmental load of pesticides was as high as 13,446 g ha^{-1} active ingredient (a.i.) in the *Yala* season. The area under cultivation in the *Maha* season is consistently higher than the *Yala* season due to the fact that ample receipt of rainfall (northeast monsoon) allows cropping in highland as well as lowland areas. Nevertheless, the lowland plots are cultivated with rice during this season with least amount of agricultural inputs (e.g. fertilizer and pesticides) and this crop requires copious amount of water. Low pesticide consumption for rice cultivation together with dry spells that deter pesticide consumption during inter-monsoonal periods (e.g. early- or late-*Maha* season) may result in lower pesticide inputs (9,988 g ha^{-1} a.i.) compared to the *Yala* season; therefore, the overall environmental load of pesticides in the

representative villages in the catchment was approximated to be 23 kg (23,434 g) a.i. ha^{-1} $year^{-1}$. The seasonal cropping patterns and agronomic practices introduced sediment into the Uma-oya and were aggravated by high amounts of seasonal rainfall in some months. The detailed results of the study are discussed in Sumith and Munkittrick (2011).

The first step in the fish assessment process was to identify a potential study design. Chapter 2 described the steps involved in developing an agricultural impacts assessment program focused on stream health, using preliminary studies that included (a) choice of monitoring level and strategy; (b) selection of fish species for monitoring; (c) timing of sampling; (d) sample size requirements; and (e) selection of appropriate reference site(s). Preliminary fish sampling data from several tributaries of the Uma-oya and the Amban-ganga (Knuckles streams) from the Mahaweli River basin were evaluated, and temporal patterns of gonadal recruitment were investigated for three common species: *Garra ceylonensis*, *Devario malabaricus*, and *Rasbora daniconius*. Since the methodologies for population level ecological risk assessments in agricultural catchments are still emerging (Brasfield 2007), and have not been practiced in tropical regions, the theoretical aspect of the Chapter 2 reliably integrated the available information, identified the data gaps, and recommended an overall approach to population risk assessment in Sri Lankan river systems. Chapter 2 also provided a summary of the criteria useful in the selection of most sensitive sentinel fish species for the evaluation of agricultural impacts in Sri Lanka.

The most important criticisms of this type of field assessment revolve around the selection of the reference sites, the potential presence of confounding factors, how to deal with natural variability, the statistical design, and potential genetic influences (Munkittrick 2009). Gray (2003) studied temperate streams in Canada over a gradient of agricultural inputs for cumulative impact and showed reduced reproductive output (reduction of young-of-the-year size classes) in resident fish slimy sculpin (*Cottus cognatus*) populations. Data indicated that the peak

risk period may occur in late summer, with chemical application coinciding with increased thunderstorm activity, rather than on overwintering populations of adult slimy sculpin with active gonad development (Brasfield 2007). The earlier studies by Gray (2003) and Brasfield (2007) sampled along a mainstem river and had treated tributary streams as if they were point source inputs for the assessment. The proposed clustered gradient design for the sampling within the catchment (from Chapter 2) assumed that agricultural stressors would impact along an increasing gradient of intensification in the catchment. It is necessary to eliminate any potential confounding factors encountered in the upstream reaches, and also to reduce the risk of overharvesting fish at the furthest upstream and furthest downstream reaches. The Knuckles streams were located in a protected forest reserve in a different catchment which was selected as a surrogate reference site. The species selection in impact assessments mainly depends on life history patterns, especially the reproductive cycle (Barrett and Munkittrick 2010). The potential of *G. ceylonensis* and *D. malabaricus* for use as sentinels in field studies in agricultural areas has not been studied despite their ecological relevance (i.e. wide distribution and abundance). Different reproductive seasons of *G. ceylonensis* and *D. malabaricus* between catchments have also been ignored and remain as a concern in impact assessment studies. Speciation and behavioural divergence in fish species is well documented (Ritchie 2007), and inter-species variability in reproductive life patterns of *Garra* spp. have been reported (Konswal 1990).

We selected the Knuckles streams as potential reference sites– that presented a number of contrasting issues. Chapter 3 described the results of a standard fish community survey of the Uma-oya and the Knuckles systems. Resident biota of aquatic ecosystems serve as continuous monitors of cumulative effects on those systems and are often used as endpoints in environmental assessments (Munkittrick et al. 2000). In fact, the present community structure is the result of the actions in the past. Few fish species, viz. *G. ceylonensis*, *D. malabaricus*, and *R.*

daniconius were present with sufficient abundance for monitoring studies within and between catchments.

This study also revealed enigmatic decline of *Puntius* spp. in the Uma-oya catchment. Earlier observations by Moyle and Senanayake (1984) implied that *P. titteya* is distributed in undisturbed streams in freshwater habitats. Species richness and composition have been integral assessment components in some of the community assessments such as index of biological integrity (IBI) (Karr 1981). The number of species in the genus *Puntius* could be one of the components one would consider in developing an IBI for agriculture impact assessments in stream systems in future studies. However, the very limited number of fish species in study sites (Chapter 3) complicates any attempts to design species composition metrics for an IBI assessment. For example, Karr (1981) reported that headwater streams in Illinois (USA), where IBI has been practiced was supported with 10–15 species, while a large midriver situation in the same watershed routinely supported twice as many species in a similar length of stream. The present study highlighted that there were higher number of endemic fish species (with a significant representation of *Puntius* spp.) with a description of new localities for two endemic fish species (*P. kamalika* and *D. pathirana*) in the Knuckles streams.

Exotic fish species are considered biotic "pollutants" in natural environments (Goren and Galil 2005; Nico 2010). Observations made in Chapter 3 from two stream catchments provide support that anthropogenically impacted situations favour exotic invasions, as has been previously stated (Kennard et al. 2005; Casatti et al. 2006). Being that the interactions of exotic and indigenous fish species are complex (Courtenay and Robins 1975), previous studies have generalized that exotic species would be destructive to the indigenous fish base and riverine species in particular (Fernando et al. 2002; Amarasinghe et al. 2006). Chapter 3 reported a case of strong inhibition of *G. ceylonensis* in a *Garra* dominant agricultural catchment in the presence of exotic *O. niloticus*.

Chapter 4 explored the potential impact of agriculture using reproductive and somatic parameters of *G. ceylonensis* populations. In conducting the assessment, we used a clustered gradient design (Chapter 2), which used reference sites in the upper catchment, as well as some comparisons with the Knuckles surrogate catchment (Chapter 4). The patterns of indicator responses in growth, development, and reproductive success of fish populations integrate the changes in the ecosystem and signal important changes in environmental conditions. The land use and drainage characteristics of the agricultural catchment were used to define two subjective assessment scales, viz. (a) contamination scale and (b) elevation scale. Spatial correlations along a stressor gradient and temporal correlation relative to a time course of exposure to the effects are among primary causal criteria in cumulative effects and ecological risk assessment (Culp et al. 2000a). Significant correlations of *G. ceylonensis* male size (total weight) to both elevation and percent agriculture in the sampling reaches in the Uma-oya imply partly, that basic assumptions of the cumulative assessment have been met. Mature individuals of *G. ceylonensis* sampled in the Uma-oya exhibited no differences within sites in gonad size (GSI), liver size (LSI) and condition (K) in both sexes in both sampling years (January 2010 and 2011). These minimal differences were sufficient to characterize that within-the-catchment variability was not drastically influencing for temporal comparisons thus allowing detecting impacts without affecting naturally occurring variability in reproduction.

In the high contamination scale, female *G. ceylonensis* had significantly smaller gonads and potentially less number of offspring despite having higher body mass and length. These observations imply that high agricultural inputs produced sub-optimum gonad investment and fecundity of females. The reduced gonadal investment may be due to impaired resource partitioning and metabolic disruption, and affected by high sediment influx. These types of responses were typical in stream ecosystems where nutrients and chemicals (e.g. pesticides) simultaneously stress organisms (Gibbons and Munkittrick 1994; Gagnon et al. 1995).

A strong eutrophication response (i.e. increased food availability) resulted in larger livers in both male and female *G. ceylonensis*, which was consistently evident in the agricultural catchment compared to the forested catchment. The contrastingly high reproductive performance in male *G. ceylonensis* was not obvious in simple site comparisons within the Uma-oya catchment, but became evident by comparison with fish from surrogate reference catchment (Knuckles) in a forested reserve. Interestingly, female fish at higher elevations produced larger gonads (high gonadosomatic index; GSI) despite their smaller body mass in the Uma-oya. Though the two assessment scales could have been confounded by catchment variables, two major responses were evident: high reproductively efficient higher elevation females (due to eutrophication) and low reproductively efficient middle elevation females (metabolic disruption or toxicity). The latter response is also observed in high contamination sites in the Uma-oya agricultural catchment with significant variation in total fecundity (Chapter 4). This study confirms previous thoughts that causality of cumulative effects can be segregated from gradient effects due to nutrient enrichment (Bussières et al. 1998). The larger size of *G. ceylonensis* in study catchments also bring a significant confounding effect by increased human exploitation (Chapter 3).

It was rather unexpected that the relative liver size of prespawning *G. ceylonensis* collected from sites located in the forested Knuckles streams, and the prespawning populations from the Uma-oya catchment were greatly different, confounding the data interpretation. With the apparent behaviour of spawning migration of the Knuckles *G. ceylonensis*, the greater liver size (high liversomatic index; LSI) in *G. ceylonensis* collected from the Uma-oya may not be showing conclusive evidence of effects (i.e. nutrient enrichment effect), though the higher GSI in male fish would provide complementary results. Some subtle cryptic morphological and reproductive differences in *D. malabaricus* populations between the Uma-oya and the Knuckles were also noted (Chapter 2 and 3). To better understand mechanisms underlying species

divergence in *G. ceylonensis* and *D. malabaricus* populations, genetic assessments are currently being conducted for full MT-CO1 (mitochondrially encoded Cytochrome Oxidase 1) regions. Because the use of homogeneous fish populations is necessary for environmental monitoring, genetic tools are required to differentiate homologous species living in river systems to provide meaningful data assessments. Additional population-level assessments were also completed on two other species, but have not been included in this dissertation due to space constraints.

The Uma-oya catchment was chronically stressed over the years by sediments and pesticides, and more intensively during the *Maha* season from November–January. The contrasting reproductive seasons in the Knuckles (November) and the Uma-oya (January) populations of *G. ceylonensis* may be due to siltation and sedimentation. The rainfall and temperature (stream and ambient air) profiles between catchments were similar between these two catchments suggesting that these environmental factors were not contributing to site differences (Chapter 2 and 4). There is evidence that fish breeding and spawning may be responsive to environmental conditions (Balon 1975, 1984), and there is also evidence that the relationship of sediment levels to fish performance is complex (Gray 2003). However, because *G. ceylonensis* is lithophilous, requiring clean gravel or rubble substrate on which to spawn (Jones 1938; Berkman and Rabeni 1987), and requires more specialized feeding from the benthic habitats (De Silva 1991), they can be more negatively impacted by siltation and sedimentation in the Uma-oya. De Silva (1991) observed the response seen with *G. ceylonensis* in the Uma-oya agricultural region over a broader geographical range in the Mahaweli River basin.

A study is not complete without exposure assessments. Chapter 5 focussed on quantifying the response of the Ceylon stone sucker, *G. ceylonensis* and two other species (*D. malabaricus* and *R. daniconius*) to exposure to cumulative agricultural contaminants with respect to specific biomarker responses. The effects-based assessment approach (Chapter 4) was based on a premise that holistic adverse effects of receiving water bodies expressed as an

"effect" can be observed at lower levels of biological organization (Munkittrick et al. 2000; Dubé and Munkittrick 2001; Dubé 2003). Most traditional toxicology studies are focused strictly on mortality imposed by toxicants (directly determining LD_{50} or LC_{50}), however there is increasing awareness of the potential for more subtle chronic effects of contaminants on fish health and performance.

Based on the pesticide use data in the Uma-oya catchment (Sumith and Munkittrick 2011), six of the most commonly used twelve pesticides are either organophosphates (OPs) or N-methyl carbamates (NMCs). According to acute and sub-acute toxicity data, OPs and NMCs represent the greatest risk to fish by acetylcholinesterase (AChE) inhibition. In addition, several non-OP and non-NMC pesticides such as glyphosate (Salbego et al. 2010) and propineb (Viviani et al. 2008), are known to modulate cholinesterase (ChE) enzyme activity in organisms. Mancozeb and chlorothalonil are fungicides most commonly applied across seasons in the Uma-oya catchment, but they have no known AChE inhibitory activity.

Pesticide applications vary during the *Yala* and the *Maha* vegetable cultivation seasons depending on the climatic conditions with more insecticide applications in June to August than during the *Maha* season. Instead, high rainfall during the *Maha* cultivation season (October to January) favours more fungicide use due to a "washout effect" of most plant sucking insects that reduces the need for most insecticide applications. High insecticide applications and pesticide-tainted water and sediment are transported to the streams during the *Yala* season and subsequently expose resident fish to agricultural stressors more severely than in the *Maha* season (Chapter 5).

I studied the concomitant response of AChE activity in the brain, muscle, and eye tissues in fish exposed to a complex mixture of contaminants in agricultural streams at sites with the highest relative abundance of fishes (Chapter 3). The *in situ* exposure assessment was promising in several aspects including (a) the inhibitory patterns were consistent with known contaminant

concentrations of neurotoxic OP and NMC pesticides which were statistically significant in all three species compared to controls, (b) the muscle AChE inhibition was protective on the brain AChE activity in muscular energetic species like *D. malabaricus* and *R. daniconius,* (c) the eye AChE inhibition was most sensitive in *G. ceylonensis* even under contaminant concentrations which were diluted and/or undetectable (subjected to method detection limits in the present study) under standard spectrometry.

The inhibitory pattern of AChE is suggested to serve as a sensitive and useful biomarker of neurotoxic contaminant exposure in agricultural catchments (Roex et al. 2003; Wijeyaratne and Pathiratne 2006; Golombieski et al. 2008). The AChE speciation in the three fish species are poorly studied except in the reported case of few cyprinid fishes that have shown only AChE activity in their muscle (Lundin 1962), and the genetic base on a closely related lineage, *Danio rerio* (Bertrand et al. 2001). *Devario malabaricus* and *R. daniconius* have been classified as larger-relative-eye-diameter-species (Bhat 2004), while *Garra* spp. (e.g. *G. gotyla*) has a smaller eye diameter. Similarly, *D. malabaricus* and *R. daniconius* have been classified as fast active cruisers in the water column (McClure et al. 2006), while *Garra* spp. (e.g. *G. ceylonensis*) is sedentary, living on the stony stream bottoms (De Silva 1991; Pethiyagoda 1991). The theoretical aspects of the different AChE activities in tissues and life history patterns were complementary (Chapter 5).

One key challenge is the understanding of the link between pesticide effects on individual fish and the intrinsic productivity of populations. Although the dissertation does not link lower reproductive potential in female *G. ceylonensis* (Chapter 4) to AChE inhibitory levels (Chapter 5) in agricultural sites, theoretical evidence may predict population impact by a cascade of events that lead to accumulated effects in the ascending order of hierarchy in biological organization (Chapter 1). For example, exposures to low, environmentally realistic concentrations of organophosphates (e.g. chlorpyriphos) resulted in reductions in AChE activity

that closely correlated to reduction in bioenergetic functions such as swimming speed and feeding rates from laboratory studies. Reductions in feeding are likely to lead to reductions in the size of gonads and fecundity an end point that has been shown to be an important determinant of ecological competence and survival in individual fish (Kumar and Chapman 2001; Dutta and Maxwell 2003; Sandahl et al. 2005). However, it is apparent that the low reproductive potential of *G. ceylonenesis* in the middle catchment may not be reflected in the population performance. In situations such as the nutrient levels have come to the point of eutrophy in streams, potential contaminant effects could be masked by organisms (Culp et al. 2000b). For example, Culp et al. (2000b,c) demonstrated that invertebrate variables below pulp and paper mill discharge points in rivers were largely responsive to nutrient enrichment rather than that of toxicity.

The present study did not address the impact of endocrine disruption as a function of pesticide exposure on fish. Nevertheless, exposure to agricultural contaminants such as pesticides may disrupt hormones and cause adverse developmental, disease, and reproductive problems (Matthiessen and Logan 1984; review by Denslow and Sepúlveda 2007). Morphological abnormalities similar to lordosis/ scoliosis were observed in three locations in the Uma-oya during January (2010) sampling (Figure 6-1). The recorded incidences were 3% (3 in 100 individuals) in Puhulpola for *G. ceylonensis* and 5% (6 in 119), 3.3% (4 in 122) and 1.1% (2 in 186) in Girambe, Medawela and Puhulpola for *D. malabaricus*, respectively. It is quite inconclusive at this stage to conclude population level significance of abnormalities because; (a) natural occurrence levels are not known for these species, and (b) similar abnormalities may occur to a variety of compound effects such as high estrogenic compounds (Boudreau et al. 2004), and physical effects such as excessive tail muscle activity against flow (Kihara et al. 2002), functional abnormalities in swim bladder (Jacquemond 2004). Several studies (Baumann and and Hamilton 1984; Colombo et al. 2005; Sumanadasa et al. 2008) reported that skeletal abnormalities in vertebrates including fish were caused by organophosphate insecticides used in

agricultural catchments. The possibility of agricultural and pesticide impact to observed malformations needs to be investigated.

6.2 Integration of dissertation chapters

Iterative study designs follow a progressive series of studies designed to achieve a goal– the goal of this study was the progressive development of information to design an assessment program, not specifically to define the causes of the changes that occurred. That being said, it is possible to use the existing data to inform the design of a study that could address the issue of causation. I do want to emphasize that attributing cause to any impacts in this study is largely speculative correlation. I do believe that a new study could be designed given the available knowledge accumulated in this dissertation, to examine the potential causes and mechanisms of the impacts observed.

The clustered design used here has two sets of potential reference sites: the Knuckles reference sites, with their inherent challenges, and the least-contaminated sites in the Uma-oya. Since chemistry data were not available for all sites, I designed a subjective ranking for site stressors based on best professional judgment and experience to identify which sites might best be used as the control sites (least-impacted) (Table 6-1). The data suggest that Perawella (PW) and Hevanakumbura (HK) would represent the least-impacted sites within the Uma-oya, and were selected as in-catchment reference sites; Rambukoluwa (KN1) and Illukkumbura (KN2) were selected as the Knuckles reference sites.

Summaries were made for individual levels measurements relative to the Knuckles values (as a % change; Table 6-2) and for the average PW/HK values) (Table 6-3) as well as key community parameters (Table 6-4). Correlations were then explored with stress summaries (Table 6-1), as well as against the percent changes (Table 6-2, Table 6-3,Table 6-4) and against ranking summaries in terms of the number of changes detected larger than the critical effects

sizes (CES) determined for Environmental Effects Monitoring (Munkittrick et al. 2009) (Table 6-5). Correlations were also explored with site elevations, and a calculation of % land under agriculture, and with stream water quality data for those sites where data were available (Table 6-6), as well as habitat variables (Table 6-6).

There were significant correlations between % land in agriculture and nitrate levels in Chapter 3 ($p = 0.03$; $r^2 = 0.72$) and phosphate ($p = 0.02$, $r^2 = 0.76$), but no relationship with elevation ($p = 0.88$) (Table 6-7). Pesticide residue data were only available for three sites, but showed no consistency with elevation, nitrates, or effect endpoints; Erabadda (ER) was by far the most contaminated with pesticides but showed amongst the lowest fish effects.

The number of pair wise comparisons to explore the data is excessive, and the comparisons are used in this case only to develop hypotheses for helping design follow-up studies. The data from the community study (Chapter 3), both total abundance and the numbers of exotic species showed statistically significant correlations with the total stress level (from Table 6-1) ($p < 0.01$), although total abundance showed stronger correlations with nitrates ($p = 0.003$) and was related to phosphates ($p = 0.016$). The number of exotic species was also correlated with *G. ceylonensis* abundance, and total abundance was correlated with species richness.

Within the individual level endpoints, the Knuckles comparisons give the most differences compared to CES (Table 6-2), but total stress showed no correlations with individual level responses. As was seen in earlier comparisons, total weight of both male and female *G. ceylonensis* correlated with elevation ($p < 0.02$) but no other correlations were seen with elevation or % land in agriculture production. The strongest correlations with condition (K) in both male and female *G. ceylonensis* were seen with dissolved oxygen levels but p values were close to 0.2 (Table 6-7). Liver size (LSI) and gonad size (GSI) in both sexes in *G. ceylonensis* showed strong correlations with nitrate, and LSIs in females was also correlated with phosphate

levels, and GSIs in both sexes also correlated with sulphate levels; the strongest correlation was between sulphate and female GSI ($r^2 = 0.86$, $p < 0.009$). Fecundity showed the strongest correlation with chlorophyll-*a* levels ($p = 0.035$).

In summary, for designing future studies, the best indications of agriculture appear to be nitrate and phosphate levels in water samples. Total stress appears to be a better indicator of potential community level responses, while the nitrate and phosphate levels correlate with many of the key fish endpoints. Fecundity represents recent energy utilization, and was correlated only with chlorophyll-*a* levels. Future studies should design a clustered gradient design, and reference sites will best be represented by the lowest stress levels within a system. Water quality levels of nitrate and phosphate could be used to design replicated sites, and to define reference locations. Caution should be used when interpreting these correlations, as they do not represent definition of cause and effect; for example female GSIs showed a strong positive correlation with nitrate levels, but the analyses in Chapter 4 suggested that GSIs in *G. ceylonensis* females are lower than that would be expected based on fish size. Additional indicators of exposure risk to pesticide residues are needed.

6.3 Significant contributions of the dissertation

Agricultural ecosystems in developing countries have been widely impacted by chemical inputs and system alterations such as channelization, land clearing and degraded riparian buffers. Current research in these countries is often limited to laboratory verifications and research on broader relevance in ecological settings is scarce. Population-level assessment of agricultural impacts using indigenous fish species is of high relevance to the public, but studies have largely been limited to temperate environments. This dissertation adapted and applied the technologies developed for environmental monitoring in Canada to stream systems in Sri Lanka. The approach focused on assessment methodologies using population impacts as the ecologically

relevant context, with attempts to integrate effects-based and stressor-based approaches, complementarily (Figure 6-2). Reducing uncertainty surrounding cumulative effects assessments in agricultural catchments in novel environments is undoubtedly a considerable scientific advancement for future study phases. I have shown in this dissertation that the life history patterns of *G. ceylonensis* are supportive for agricultural impact assessments in the tropical south and southeast Asia. That is, general indicators such as abundance, growth, feeding habits, reproductive strategies (i.e. single and total spawner), and spawning time were complemented with the exposure periods and stressor patterns. There are supportive evidences in this study to make diagnostic inferences about ecosystem impacts by a sequential order of response to exposure to agricultural pollutants within a biological system. Thus, one of the most important advantages recommending development and elucidation of effects is to develop an understanding of underlying mechanisms to diagnose the impact of agricultural stressors, and thereby help protecting ecological resources by suitable management and regulatory tools.

6.4 Final considerations

This research study is one of the first to develop a fish population assessment program for tropical agricultural environments. There are important differences between tropical and temperate environments in (1) climate related parameters, (2) ecosystem sensitivity, and (3) agricultural practices (Daam and Van den Brink 2010). In temperate climates, there have been considerable advancements in research on the biological effects from non-point source agricultural inputs (e.g. Gray 2003; Ibarra et al. 2005; Brasfield 2007; Finley 2008; Smedley 2009). The cumulative effects assessment requires a *prima facie* understanding of a broader biology of fish species before integrating the use of multiple assessment tools (e.g. stable isotopes, biomarkers, DNA fingerprinting) in studies on agricultural impact.

The following list represents significant questions and research needs related to tropical impacts of agriculture, including;

(1) More information on the basic ecology and biology (e.g. spawning migrations) of the Ceylon stone sucker (*Garra ceylonensis*) and other small-bodied fish species will aid in their use as a sentinel species for environmental monitoring and for the interpretation of results and responses of these systems to environmental stressors.

(2) Studies should be conducted on the mobility of *G. ceylonensis* in different catchments, including using stable isotopes. The variability in isotopic values could be measured both spatially and temporally because the isotope values are based on trophic status, movement into other locations or migratory behaviours that would result in a more variable signature. Studies could iteratively incorporate environmental variables (i.e. temperature, habitats, dissolved oxygen) in such assessments. Site fidelity is an important component in fish species selection for environmental monitoring (Munkittrick 2009).

(3) Comparison of effects-based and stressor-based responses of fish species to determine whether the current fish species are adequately reflecting the responses of other biota exposed to the same stressors.

(4) Application of methods to evaluate sedimentation and siltation in different catchments to allow for meaningful comparisons of impacts on reproductive and other indicators (i.e. food habits) to resident biota. The timing and methodology of these tools needs to be adaptive and refined to be supportive in answering the site- and study-specific questions raised by the biological responses observed.

(5) Studies on characterization of AChE species in different tissues in the common fish species would facilitate selection of the most sensitive fish species and most sensitive tissues for environmental monitoring. Use of exotic fish species for impact assessments does not provide representative data and/ or real hazard for indigenous species (Wijeyaratne and Pathiratne 2006; Daam and Van den Brink 2010).

(6) Improved understanding is needed of the pathways of pesticides from the field to surface/ ground waters from agricultural activities (particularly during events of runoff and sediment transport to adjacent streams). Assessment methods could be improved by isotope tracers from fields adjacent to agricultural fields and in-stream biota at each level of the food web in order to trace the sources of effects on growth and productivity.

(7) Gibbons and Munkittrick (1994) recommended that iterative (i.e. adaptive) studies are required to examine for fish showing specific responses of reproduction, metabolic redistribution and other indicators of reproductive health by a detailed physiological investigation of energy expenditure and storage and hormonal regulation of reproduction and growth in relation to agricultural exposures.

(8) Histological examinations during various stages of gonad maturity may help in determining when the critical effects are initiated in mature fish.

A consortum of efforts are required to develop an effective effects-based assessment of agricultural impact, relying on iterative study designs and new assessment tools. A considerable understanding has been accumulated on contaminant interactions and confounding influences in

cumulative effects assessment studies over the past several years. Biological response patterns to contaminant exposure seem to be invariably common across studies, nevertheless the challenge in each study is to effectively understand the ecosystem components and strategically remove relevant modifiers, using study designs and suitable statistical methods in order to "tease out" real impacts occuring in the field. Several of these endpoints have formally entered into any ecological risk assessment (ERA) framework.

The objective of the dissertation was to design a fish assessment program for high pesticide use agricultural areas in Sri Lanka. The steps were dispersed during the development of the research but have been linked in Table 6-8. The basic steps include defining the scope and scale of the issue, selecting an appropriate study area, characterizing the environment, selecting key sites, the sampling strategy and developing the study design. These steps are followed by the preliminary data collection, finalizing the sampling design, data analysis, and verification. Any good assessment requires a series of iterative studies to focus in on the causes of the changes.

This study concluded that there were potential eutrophication effects at higher elevations and chemical impacts at lower elevations but there were interactions between nutrients and elevation. Potential next steps would include studies on (a) sub-catchment(s) with known/correlated farming activities to sample before/after, with wild fish sampling before and after application of pesticides, including potential mesocosm/cage studies, (b) studies along a gradient of both nutrients at the same elevation and at different elevations with similar nutrient levels, (c) investigations of potential genetic influences on performance, and (d) confirmatory studies using benthic macroinvertebrates. These studies would help identify the subsequent studies to confirm impacts and would represent a comprehesnsive approach for assessing high pesticide use agricultural areas.

6.5 References

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Table 6-1. Subjective estimates of relative stress for sites in the Uma-oya. Values for width, depth and upstream stress are relative values; others are given in footnote*. For descriptive site codes, see Figure 4-1 legend. KN1 = Rambukoluwa (RO), KN2 = Ilukkumbura (IK)

Site	Width	Depth	Upstream stress	Gradient	Riparian	Substrate	Agriculture	Proximity to cities	Sand mining	Total Stress	Stress[1]	Stress[2]
HK	1	1	1	1	1	1	1	1		8	5	3
PW	1	1	1	2	1	2	1	2		11	6	4
GB	1	1	1	2	2	2	1	1	1	12	8	5
ME	3	1	2	3	3	3	3	2		20	14	9
ER	2	2	2	3	3	3	3	2	1	21	14	9
RT	2	1	2	2	3	2	3	2		17	12	8
DT	1	1	1	2	2	2	1	2		12	7	5
GI	2	2	1	3	3	3	3	2		19	12	9
MT	3	2	3	2	3	3	3	2	1	22	15	8
PU	3	3	3	1	3	3	3	3		22	13	7
RP	2	2	2	1	3	3	2	1		16	10	6
MD	3	2	3	3	2	2	2	1		18	13	7
KN1	1	3	2	2	1	1	1	1		12	8	4
KN2	1	3	2	2	1	1	1	1		12	8	4

*

Rank	Width/ Depth	Stress	Gradient	Riparian	Substrate	Agriculture
1	Low	Low	Low	Good	Small	High
2	Medium	Medium	Medium	Medium	Medium	Medium
3	High	High	High	Bad	Large	Low

[1] – Combination of sand mining, upstream stress, width, gradient, riparian, agriculture

[2] – Combination of gradient, riparian, agriculture

Table 6-2. Absolute percentage difference from reference for the key endpoints for *Garra ceylonensis*, using the Knuckles sites as the reference. Highlighted values reflect differences the critical effect sizes defined for Environmental Effects Monitoring (Munkittrick et al. 2009); (↑) represent increases and (↓) represent decreases relative to the reference. For descriptive site codes, see Figure 4-1 legend. KN = Pooled data of KN1 =Rambukoluwa (RO) and KN2 = Ilukkumbura (IK).

Knuckles Reference	Females					Average Difference	Males				Average Difference
Site	Weight (g)	K (%)	LSI (%)	GSI (%)	Fecundity	Females	Weight (g)	K (%)	LSI (%)	GSI (%)	Males
PW	37.5	8.3	103.1↑	33.3↓	30.6↓	45.55	68.6↓	5.0	111.3↑	37.5 ↑	55.6↑
HK	14.1	1.5	114.1↑	18.1	4.9	36.94	10.9	10.0↑	87.3 ↑	80.2 ↑	47.1↑
GB	33.5	3.0	40.6↑	35.8↓	42.7↓	28.21	39.4↓	11.7↑	26.8 ↑	49.4 ↑	31.8↑
ME	33.9	0.0	32.8↑	6.2	0.1	18.21	28.0↓	8.3	12.7	57.4 ↑	26.6↑
ER	29.0	1.5	92.2↑	19.0	8.4	35.43	26.3↓	14.2	54.9 ↑	39.6 ↑	33.7
RT	23.8	0.8	75.0↑	23.5	35.9 ↑	30.75↑	18.9	8.3	50.7 ↑	41.4 ↑	29.8↑
DT	22.6	3.8	87.5↑	29.1↓	3.4	35.72	27.4	8.3	59.2 ↑	35.7 ↑	32.7↑
GI	5.2	6.0	79.7↑	4.5	11.9	23.85	26.3	8.3	76.1 ↑	68.5 ↑	44.8↑
MT	5.7	3.8	103.1↑	48.0↓	4.8	40.14	30.9	17.5↑	62.0 ↑	52.2 ↑	40.6↑
PU	41.9	6.0	62.5↑	17.9	33.7↑	32.08↑	11.4	5.8	78.9 ↑	47.8 ↑	36.0↑
RP	62.1	0.8	64.1↑	11.7	60.1↑	34.66↑	36.0	7.5	52.1 ↑	66.7 ↑	40.6↑
MD	19.0	8.3	29.7↑	53.1↓	11.6	27.50	53.1↑	5.0	5.6	14.2	19.5
KN	-	-	-	-	-	-	-	-	-	-	-

Table 6-3. Absolute percentage difference from reference for the key endpoints for *Garra ceylonensis*, using the PW and HK as the reference sites. Highlighted values reflect differences the critical effect sizes defined for Environmental Effects Monitoring (Munkittrick et al. 2009). The average difference reflects on K, LSI and GSI results. (↑) represent increases and (↓) represent decreases relative to the reference. For descriptive site codes, see Figure 4-1 legend. KN = Pooled data of KN1 =Rambukoluwa (RO) and KN2 = Ilukkumbura (IK).

PW and HK as Reference	Females					Average Difference	Males				Average Difference
Site	Weight (g)	K (%)	LSI (%)	GSI (%)	Fecundity	Females	Weight (g)	K (%)	LSI (%)	GSI (%)	Males
PW	15.8	3.6	2.6	10.2	15.6	8.0	47.9↓	2.3	6.0	13.4	17.4
HK	15.8	3.6	2.6	10.2	15.6	8.0	47.9↑	2.3	6.0	13.4	17.4
GB	10.3	2.0	32.6↓	13.5	30.3↓	14.6	0.5	3.9	36.4↓	6.0	11.7
ME	10.9	5.1	36.3↓	26.3	21.7	19.7	19.4	0.8	43.5↓	0.9	16.1
ER	4.4	3.6	7.9	9.0	11.3	6.2	22.3	6.2	22.3	12.1	15.7
RT	66.9	5.9	16.1	3.0	65.2↑	23.0	34.6	0.8	24.4	11.0	17.7
DT	4.4	1.2	10.1	4.5	17.4	5.0	20.4	0.8	20.1	14.6	14.0
GI	27.7	1.2	13.9	28.6	36.1↑	17.8	22.3	0.8	11.7	6.0	10.2
MT	27.2	1.2	2.6	30.1	15.8	15.3	14.7	9.3	18.7	4.2	11.7
PU	91.3	1.2	22.1	10.5	62.5↑	31.3	84.8↑	1.6	10.3	7.0	25.9
RP	118.5	4.4	21.4	18.8	94.7↑	40.7	6.2	0.0	23.7	4.9	8.7
MD	9.2	3.6	37.8	36.8	7.4	21.9	154.0	2.3	52.7	28.0	59.3
KN	34.8	5.1	52.1	34.6	21.6	31.6	65.9	7.0	49.8	37.1	39.9

Table 6-4. Community data from Chapter 3. For descriptive site codes, see Figure 4-1 legend.

Site	Abundance (Density) /m^2	Species Richness	Number of Exotic Species	*Garra* Abundance (Density) /m^2	*Devario* Abundance (Density) /m^2	*Rasbora* Abundance (Density) /m^2
PW	0.01	2	0	0.01	-	-
HK	0.03	2	0	0.03	-	-
GB	0.17	4	0	0.04	0.12	0.02
KG	0.09	4	1	-	-	0.08
ME	1.06	6	1	0.41	0.66	-
ER	0.48	6	1	0.31	-	0.15
RT	0.14	5	0	0.0	0.06	0.01
DT	0.24	4	0	0.04	0.07	0.13
GI	0.62	7	2	0.24	0.36	0.01
MT	0.34	8	2	0.10	0.19	0.06
PU	0.63	9	1	0.19	0.39	0.02
RP	0.31	5	0	0.06	0.23	-
MD	0.23	5	0	0.05	0.06	-

Table 6-5. Interpretation of individual level differences relative to reference site. "Times>CES" reflects the comparisons where the differences are greater than the critical effect sizes as defined for Environmental Effects Monitoring (Munkittrick et al. 2009; 25% for endpoints other than 10% for condition). For descriptive site codes, see Figure 4-1 legend. KN = Pooled data of KN1 = Rambukoluwa (RO) and KN2 = Ilukkumbura (IK).

Site	Times >CES Females Knuckles Reference	Times >CES Females PW and HK as Reference	Times >CES Males Knuckles Reference	Times >CES Males PW and HK as Reference	Times >CES Both Sexes Knuckles Reference	Times >CES Both Sexes PW and HK as Reference
PW	4	0	3	1	7	1
HK	1	0	3	1	4	1
GB	4	2	4	1	8	3
ME	2	2	2	1	4	3
ER	2	0	4	0	6	0
RT	2	2	2	1	4	3
DT	2	0	3	0	5	0
GI	1	3	3	0	4	3
MT	2	2	4	0	6	2
PU	3	2	2	1	5	3
RP	3	2	3	0	6	2
MD	2	2	1	3	3	5
KN	-	4	-	3	-	7

Table 6-6. Site data for the sampling locations in the Uma-oya. The % land in agriculture was calculated based on quantitative estimates using Google Earth photos taking at an elevation of 2 km. For descriptive site codes, see Figure 4-1 legend.

Site	Elevation (m)	% Land in Agriculture	Rank for Land in Agriculture	Pesticide Levels	Rank Pesticide
PW	1,187	1.8	14	NA	-
HK	1,117	91.7	1	NA	-
GB	1,101	56.8	5	NA	-
ME	1,110	84.1	2	38	3
ER	1,106	73.0	3	213	1
RT	1,081	22.2	13	NA	-
DT	1,058	26.5	12	NA	-
GI	1,054	69.3	4	46	2
MT	1,030	43.1	11	NA	-
PU	981	47.2	7	NA	-
RP	778	43.9	10	NA	-
MD	556	51.4	6	NA	-

NA = Data not available

Table 6-7. Strongest correlations between factors in pair-wise correlations.

Level	Parameter	Factor	r^2	p	Factor	r^2	p	Factor	r^2	p
Community	Abundance	Nitrate	0.87	0.003	Phosphate	0.72	0.016	Stressors	0.47	0.01
	Species richness	Abundance	0.45	0.01						
	Exotic fish	Stressors	0.52	0.009	*Garra*	0.39	0.03			
	Garra abundance	Phosphate	0.81	0.006	Nitrate	0.69	0.02			
Population	Female>CES	% land agriculture	0.32	0.064						
	Female W	Elevation	0.75	0.017						
	Female K	DO	0.41	0.17						
	Female LSI	Nitrate	0.71	0.036	Phosphate	0.87	0.006			
	Female GSI	Sulphate	0.86	0.009	Nitrate	0.81	0.015	Phosphate	0.65	0.052
	Fecundity	Chlorophyll-*a*	0.71	0.035						
	Male W	Elevation	0.46	0.014						
	Male K	DO	0.34	0.22						
	Male LSI	Phosphate	0.65	0.053						
	Male GSI	Nitrate	0.61	0.065	Sulphate	0.59	0.076	Chlorophyll-*a*	0.56	0.085
Habitat	Nitrate	% land agriculture	0.73	0.03						
	Phosphate	% land agriculture	0.76	0.02						
	Sulphate	Nitrate	0.77	0.02						

Table 6-8. An effects-based framework for fisheries assessments for high pesticide use agricultural areas in Sri Lanka.

Step	Focus	Section
1. Define scope and scale of the issue	Examine cropping patterns, pesticide use patterns, quantities, timing (define stressors of concern)	Sumith and Munkittrick (2011)
2. Select study area	Prioritize potential study areas based on cropping patterns, confounding factors and suitability for developing an interpretable study design	Sumith and Munkittrick (2011)
	Define existing data availability	
	Identify existing related studies (if any)	
3. Characterize environment	Characterize spatial, nutrient, hydrological and temporal regimes within the study area	1.6.1
4. Selecting key sites	Evaluate potential sites and timing of sampling based on stressor patterns	1.6.1
	Select appropriate off-site reference sites	2.3.1
	Select in-catchment reference sites (if available); if not, select least-impacted sites	2.8
5. Sampling strategy	Develop sampling strategy and selection of sampling methods	2.7
6. Develop study design	Examine distribution of stressors for selecting mains study design (gradient, clustered gradient, radial, control: impact, reference condition, etc)	2.9
	Initial community sampling to select species availability and variability	Chapter 3
7. Preliminary data collection	Collection of data on fish species present and abundance and life history characteristics at a range of impacted and potential reference sites	Chapter 2
	Select potential sentinel species	2.4
	Initial sampling of potential sentinel species to determine optimal timing for sampling based on reproductive development	2-5–2.7
8. Sampling design	Develop statistically-based design	4.3
	Selection of reference sites for best comparison with test sites	2.8
9. Data analysis	Analyse data using both in-catchment and regional reference site approaches	4.4
	Analyse impaired aspects and potential causal factors	6.2
	Confirm exposure	Chapter 5
	Confirm species identity, genetic relationships between sites	Unpublished data
10. Verification	Alternate catchment studies using same species and different organisms (e.g. invertebrates)	
11. Iterative steps	Revise indicators, methodology and triggers, if necessary	6.4
	Evaluate adequacy of assessment model and study design	
	Design of follow-up studies to focus in on potential impacts	
	Repeat sampling to focus in on potential mechanism of impact	

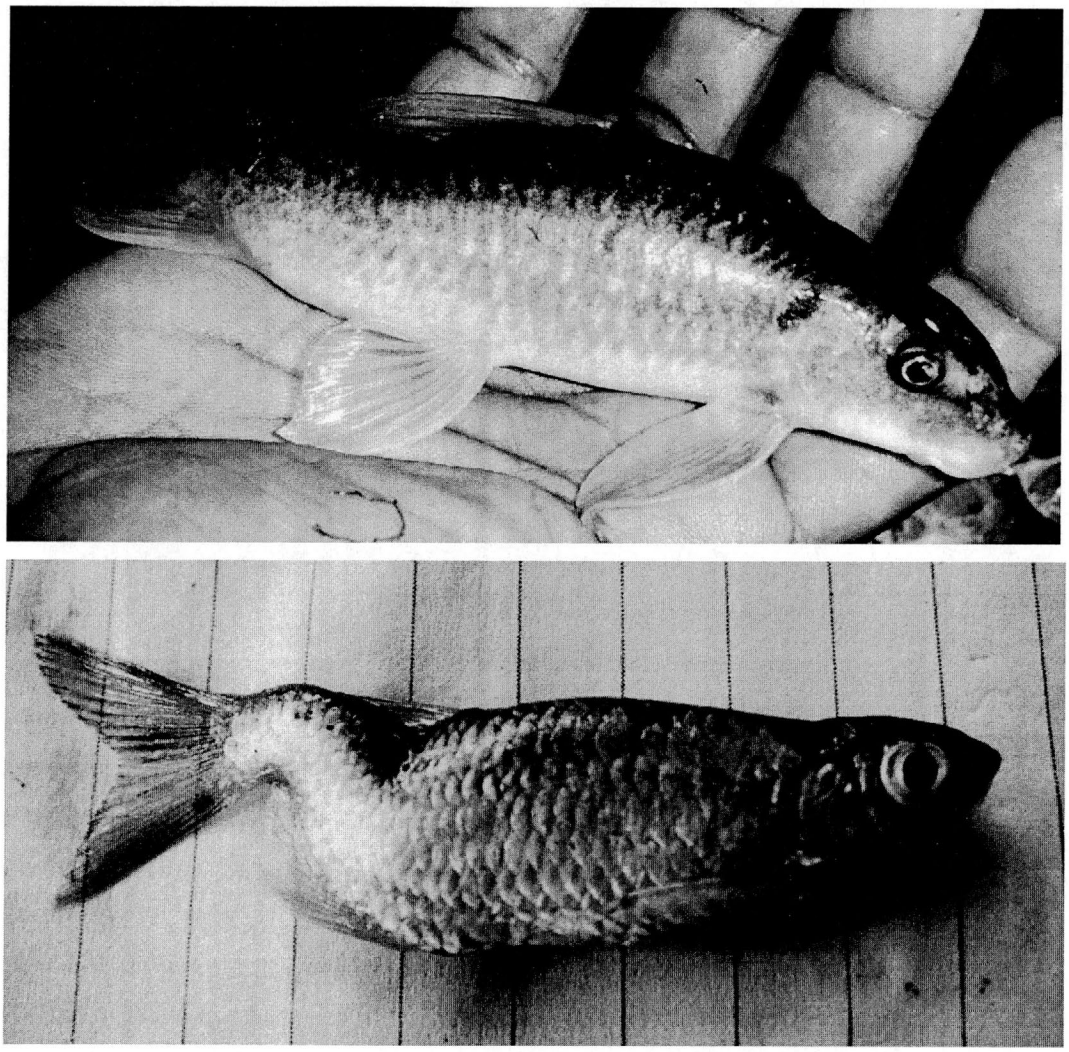

Figure 6-1. Lordosis/ scoliosis incidence observed in *Garra ceylonensis* (above) and *Devario malabaricus* (below) in the Uma-oya stream during January (2010) sampling (Photos by Jayakody A. Sumith).

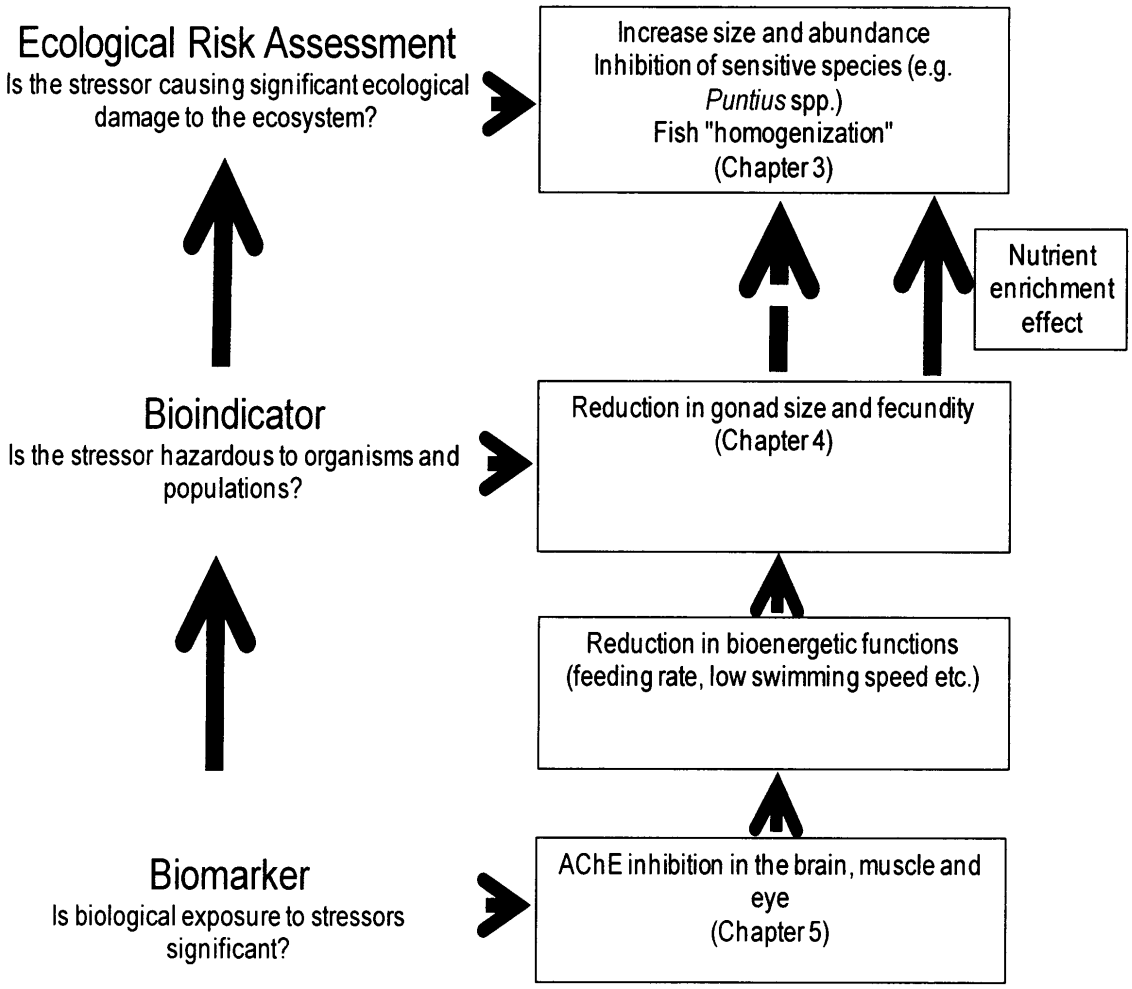

Figure 6-2. Unified schematic relationship of stressor- and effects-based agricultural impact using *Garra ceylonensis*.

7 Vitae

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