MATERIALS
AND
METHODS
3. MATERIALS AND METHODS

3.1. SAMPLING SITE AND SAMPLE COLLECTION

Fish samples were collected from river Ganga at Narora (27°30’N, 78°25’60E), U.P., India (Figure 1). The river Ganga rises in the Gangotri glacier (30°54’N; 78°54’E) in the Himalayas at an altitude of 7010 meter above mean sea level in the Uttarkashi district of Uttarakhand, India. It travels along the five Indian states (viz., Uttarakhand, Uttar Pradesh, Jharkhand, Bihar and West Bengal) before entering into the Bay of Bengal.

The study material consisted of the fish species, *Channa marulius*, *Channa punctata*, *Labeo bata*, *Hypophthalmichthys molitrix*, *Mastacembelus armatus*, *Heteropneustes fossilis*, *Clarias gariepinus*, *Clarias batrachus*, *Wallago attu* and *Ompok pabda*. Fishes were sampled using gill nets/cast nets/drag nets of various mesh sizes during the period January 2010 to February 2012. Samples of *C. marulius* were collected during July 2008 to December 2009. Samples of *Clarias gariepinus* were collected monthly from the local fish market at Aligarh, U.P., India. The collected fishes were identified according to the description given by Talwar and Jhingran (1991), Jayaram (1999) and rechecked against FishBase database (Froese and Pauly, 2012).
3.2. BODY MEASUREMENT

Total Length (TL) of each fish was measured from the tip of snout to the longest fin ray of the caudal fin. Standard Length (SL) was measured from the tip of snout to the base of caudal fin. Fork Length (FL) was measured from the tip of snout to the posterior end of the middle caudal fin rays. All fish samples were measured to the nearest mm. Body weight was recorded as Total Weight (TW) including gut and gonads to the nearest 0.1 gm.

3.3. PRECISION OF AGE ESTIMATES
The study of age estimates was undertaken following the methods described by Tandon and Johal (1996), Khan and Khan (2009), Khan et al. (2011a) and Khan et al. (2011b).

3.3.1. Collection and preparation of scales

The fishes were washed under running tap water by gently rubbing their body in a head-to-tail direction in order to remove any loose scales which may have rubbed off other fish. For age estimation, scales were removed using forceps from above the lateral line near the tip of the pectoral fin. Scales were washed, cleaned and studied as dry mounts, after removing the extraneous matter and mucous by washing them in tapwater and rubbing in between the finger tips.

i) *Hypophthalmichthys molitrix*

Scales were washed in tap water and dried on a filter paper. Five to eight scales from each fish were mounted dry between two glass slides, fastened each end by means of cello tape and examined for age reading.

ii) *Channa marulius* and *Labeo bata*

To make more clear and soft (in case of large scales), the scales were dipped in weak solution (1%) of potassium hydroxide (KOH) for about 5-10 min, then washed in tap water and dried in air. Small sized scales were mounted between two glass slides and studied with the help of compound microscope (Tandon and Johal, 1996).

iii) *Channa punctata*

Scales were immersed in 5% KOH for about 5 min, washed, dried, arranged between two glass slides for microscopic observation (Reddy, 1981).
3.3.2. Collection and preparation of opercular bones

i) *Channa marulius, Hypophthalmichthys molitrix, Labeo bata and Ompok pabda*

The opercular bones were detached with the help of scalpel and dipped in boiling water for few minutes to remove extraneous tissue. A bristled brush was used to clean the opercular bones. They were then examined under transmitted fluorescent light with naked eye (Phelps et al., 2007).

ii) *Channa punctata*

Opercular bones were dipped in boiling water for few min to remove extraneous tissue, and a bristled brush was used to further clean the bones. They were then placed in ethyl alcohol for 5 min, dried, and examined under microscope.

iii) *Mastacembelus armatus*

Opercular bones were put into boiling water for 2 to 3 min, rinsed with tap water and air-dried. Opercular bones were examined dry with transmitted light and in water with reflected light on dark background (Sipe and Chittenden, 2001).

3.3.3. Collection and preparation of otoliths

To obtain otoliths, an incision was made on the dorsal side of the head, to expose the brain on either sides of which the otic capsules are located. Sagittal otoliths (largest of their kind) removed from otic capsules by opening the otic bulla, were used for age reading.

i). *Channa marulius and Channa punctata*
Otoliths were washed, cleaned and read whole by immersion in glycerol. Otoliths with unclear annual rings were ground with sandpaper to make the annuli more distinct and examined under microscope using reflected light (Tandon and Johal, 1996; Khan and Khan, 2009).

ii). *Labeo bata* and *Hypophthalmichthys molitrix*

Otoliths were washed, cleaned and read by placing them in alcohol. Otoliths with unclear annual rings were ground with sandpaper to make the annuli more distinct and examined under microscope using reflected light (Tandon and Johal, 1996; Khan and Khan, 2009).

iii). *Clarias gariepinus*, *Heteropneustes fossilis* and *Wallago attu*

Sagittal otoliths were removed, cleaned, immersed in ethanol, and examined under microscope in whole view on a black background. Otoliths with unclear annual rings were ground with sandpaper to make the annuli more distinct for age reading (Tandon and Johal, 1996; Khan et al. 2011b).

iv). *Clarias batrachus*

Otoliths were washed, cleaned and read whole by immersion in 50% glycerol and observed under microscope using reflected light.

v). *Mastacembelus armatus*

Otoliths were removed from the fish, rinsed in distilled water to remove any tissue, dried, immersed in xylene and observed under microscope. Otoliths with unclear annual rings were polished with sandpaper to make the annuli more distinct.

vi). *Ompok pabda*
Whole otoliths were submerged in a petri dish with black base filled with water and viewed under microscope with reflected light.

3.3.4. Collection and preparation of vertebrae

i) *Channa punctata, Channa marulius, Labeo bata and Hypophthalmichthys molitrix*

Vertebrae (4\textsuperscript{th} to 10\textsuperscript{th}) were placed in boiling water for 10–15 min to clear the attached muscles and then dried for 2 weeks, after which annual rings were examined under microscope (Phelps et al., 2007).

ii) *Clarias gariepinus, Clarias batrachus, Heteropneustes fossilis, Ompok pabda and Wallago attu*

Vertebrae were placed in boiling water for 2-3 minutes to remove soft tissue, cleaned, air dried, and examined in xylol under microscope for age reading (Yalcin et al., 2002).

iii) *Mastacembelus armatus*

Vertebrae were placed in boiling water for 10 min to clear attached tissues, immersed in 1\% H\textsubscript{2}O\textsubscript{2} (Hydrogen peroxide) for 24 hours, dried and then examined under dissecting microscope (Ma et al., 2010).

3.3.5. Collection and preparation of fin rays / spines

i) *Channa marulius*

Dorsal fin was immersed in boiling water for a few minutes to moisten the attached flesh facilitating the removal of rays from the fin. Fin rays were cleaned with water and left to dry completely. Dorsal fin rays were sectioned with jeweller’s saw. Sections were placed on a microscopic slide and viewed under microscope (Phelps et al., 2007). Cross-sections were coated with glycerol to increase the distinctness of annuli.
ii) *Clarias gariepinus, Clarias batrachus, Heteropneustes fossilis, Mastacembelus armatus, Wallago attu* and *Ompok pabda*

Pectoral fin spines were removed at the point of articulation, air-dried and sectioned using a jeweler’s saw. If required, some of the sections were polished with sandpaper, and a drop of immersion oil was used to improve the clarity. Sections were placed on a microscope slide and aged under microscope (Buckmeier et al., 2002).

### 3.3.6. MEASURES OF PRECISION

All the otoliths, scales, vertebrae, opercular bones and fin rays/spines sections were aged independently by two readers’ without the knowledge of fish length, weight and date of collection. Such data was utilized to calculate the precision of age estimation between the two readers. However, consensus data (from the two readers for the specific structures) was utilized to compare the age estimates between structures and also to evaluate the statistical significance among the mean age estimates from different structures. A consensus between readers was required in cases where structures exhibited disagreement in age assignments by the readers (Khan and Khan, 2009). In order to produce information on measures of precision, the age estimates were subjected to the appropriate calculations and statistical treatments (Khan and Khan, 2009). Precision was measured by calculating the percentage of agreement (PA), coefficient of variation (CV), and average percentage of error (APE; Beamish and Fournier 1981) between the readers and between the pairs of ageing structures. APE is sensitive not only to age disagreement but also to the magnitude in the difference in age assignment between or among readers. APE was calculated using the formula presented by Beamish and Fournier (1981):

$$APE = \frac{1}{R} \sum_{i=1}^{R} \left| \frac{x_i - \bar{x}}{\bar{x}} \right| \times 100,$$
where $x_{ij}$ is the $i$th age determination of the $j$th fish, $x_j$ the average age calculated for the $j$th fish and $R$ the number of times each fish is aged. The coefficient of variation (Campana, 2001) was calculated as the ratio of standard deviation over the mean, and can be written as

$$CV_j = 100\% \times \sqrt{\frac{\sum_{i=1}^{R} (X_{ij} - X_j)^2}{R - 1}} / X_j,$$

where $CV_j$ is the age precision estimate for the $j$th fish.

Both APE and CV have been widely used as statistically sound measures of ageing precision in fishes (Campana, 2001). Percent agreement, although used as an index of ageing precision in fish by many researchers (Welch et al., 1993; Hoxmeier et al. 2001; Stolarski and Hartman, 2008; Koch et al., 2009), is not considered as a suitable measure of precision by several authors (Beamish and Fournier, 1981; Chang, 1982; Campana et al., 1995). Percent agreement may be expressed as the percentage of the number of observations showing similar age estimates to the total number of observations on age estimates. Percent agreement was calculated using the “Templates for calculating ageing precision” by Sutherland (2006). Age bias graphs (Campana et al., 1995) were constructed to examine potential biases between the readers. The age bias graphs were plotted between estimates of one age reader vs. another, and can be interpreted through the line of equivalence between readers. However, in this representation, the age readings of reader 2 are presented as the mean age and 95% confidence interval corresponding to each of the age categories reported by reader 1. The intent of the confidence intervals was not to assign statistical significance to the comparison, but to allow informed interpretation of any differences between the observed values to the equivalence line. Age readings from each alternative structure were paired with the best ageing structure to calculate PA, APE, and CV.
Mean age readings (consensus data) obtained from various bony structures were subjected to one-way analysis of variance (ANOVA) followed by Duncan’s multiple range test (DMRT) (Gomez and Gomez, 1984) in order to explain whether the readings from different bony structures of the same species showed significant differences among themselves (Khan and Khan, 2009). Although the mean age estimate is not an indicator for the reliability of ageing structure, it may provide useful information regarding over or underestimation of age by a structure irrespective of fish size class. This may prove useful in selecting the structure(s) that may give statistically indifferent readings when size class is not taken into account.

3.4. GROWTH ESTIMATION

The von Bertalanffy (1957) growth function (VBGF) was employed to represent the growth of specimens and fitted to length-at-age data obtained from the most suitable ageing structure for each species. The VBGF is represented as:

\[ L_t = L_\infty (1-e^{-k(t-t_0)}) \]

Where
- \( L_t \) = total length (cm) of fish at age \( t \)
- \( L_\infty \) = asymptotic mean length
- \( K \) = is a rate constant that determines the rate at which \( L_t \) approaches \( L_\infty \)
- \( t \) = time or age of the fish; and
- \( t_0 \) = the hypothetical age at which the fish had zero length.

Comparison between observed and calculated mean length-at-age (VBGF) were tested using Student’s t-test (Zar, 1996) for all the selected fish species. The back-calculated lengths (VBGF) at estimated ages between non-lethal ageing structure (scales or spines/rays) and the most suitable ageing structure in each of the selected species were compared using Student’s t-test.
3.5. LENGTH-WEIGHT RELATIONSHIP

The measurement for length and weight were taken as described elsewhere (Materials and methods 3.2). For each fish species, the length-weight relationship was calculated and described using the equation \( W = a \cdot TL^b \), where \( W \) is the body weight (gm), \( TL \) is the total length (cm), \( a \) is the intercept and \( b \) is the regression coefficient (Froese, 2006). For each fish species, the parameters \( a \), \( b \) and \( R^2 \) (coefficient of determination) was estimated by least square regressions analysis of the logarithm-transformed LWR expression, \( \log BW = \log a + b \cdot \log TL \) (Garcia, 2010). The coefficient of determination \( (r^2) \) was used as an indicator of the quality of the linear regression (Scherrer, 1984). The \( b \) value provides information on the fish growth type.

3.6. LENGTH-LENGTH RELATIONSHIP

Relationships between (i) total length (TL) v/s standard length SL (ii) standard length (SL) v/s fork length (FL), and (iii) fork length (FL) v/s total length (TL) were estimated with linear regression (Soomro et al., 2007).

3.7. CONDITION FACTOR

The degree of well-being or relative robustness of the fish is expressed by coefficient of condition (also known as condition factor or length-weight factor). Variations in the fish’s coefficient of condition primarily reflect the state of sexual maturity and degree of nourishment. Condition factor values may also vary with fish age, and in some species, with sex. It is represented by the letter \( K \).
\[ K = 100 \frac{W}{L^b} \]

Where

\( W \) = weight of fish in grams
\( L \) = total length of fish in centimeters
\( b \) = value obtained from the length-weight equation

All statistical analyses were done using MS-Excel and SPSS (version 12.0 and version 16.0).