Geometric morphometric analysis of body shape and size variations in hatchery-reared larvae of a tropical damsel fish, *Dascyllus carneus* Fischer, 1885

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Abstract

The growth trajectories of the fishes during their early developmental phase are not adequately understood due to the continuous changes in their morphology, behaviour, and feeding habits. Understanding these interactions in relation to the habits, habitat, rate of growth, rate of development, and duration of different stages of growth are useful in predicting the direction of ecological and evolutionary changes in the life history of fishes. Using, geometric morphometric methods, we investigated the stage-specific phenotypic plasticity of *Dascyllus carneus* (cloudy damsel) in captivity during the entire larval development and metamorphosis. Each developmental stage has its unique shape and size, showing significant phenotypic plasticity ($p < 0.001$). The allometric analysis showed that *D. carneus* did not change its linear pattern of allometric growth trajectory till the stage of postflexion. In contrast, non-allometric growth analysis indicated a sudden shift from negative to positive allometric growth after the postflexion stage. Based on the results, it can be concluded that both in allometric and non-allometric analyses, except the yolk sac stage, all the other post-embryonic stages followed allometric growth patterns. Apart from the stage-specific phenotypic plasticity, the similarity and dissimilarity of each developmental stage were validated using statistical methods, such as discriminant function analysis (DFA), two-block partial least square (2B-PLS), and canonical variate analysis (CVA). All stages were seen distributed into a unique trait-based morphospace as each stage contained unique shape and size features. In our study, 2B-PLS was used to unveil the head and trunk integration at each stage. We establish that, the development of head and trunk are in separate morphological modules after the yolk sac and preflexion stages. From the overall analyses, all stages, except the juvenile stage, were observed to show high level of heterogeneity and at the transition stage, well-adapted individuals were observed. Stage-specific analysis and understanding of stage-specific requirements of developing larvae will help to surpass some challenges in larval rearing practices, which are discussed in this paper.

Introduction

The wild collection and trade of marine ornamentals are almost stagnant due to ecological concerns. Though captive breeding is the major alternative option to meet the ever-increasing market demand, it is difficult in case of reef fishes due to complexities in their controlled reproduction and larval development. The most difficult phase is that of larval metamorphosis, where the morphometric complexities reflect the adaptations of the larvae to the dynamic marine ecosystem and survival strategies. These morphological changes can also give insight into the strategies that need to be followed in the hatchery production. However, the current research is not adequate and many complexities are yet to be explored^[1–10].

The growth transformations and modifications that fishes undergo make them suitable to cope with and surpass all the environmental challenges which help in their final survival confirms the hypothesis that the morphological development and growth patterns during early life stages are in accordance with the timely needs of the larvae. The dynamics involved in the process of timely change from a primitive larva to a well-adapted adult-form suggests that survival is the prime purpose for these impeccable changes^[11–17]. During the larval development, many critical periods have been observed, which remain as obstacles during

the larval rearing. These critical stages are highly correlated with ontogenetic events such as preflexion, flexion, transition, and metamorphosis. Tracing the patterns of growth and identifying the critical periods of development would eventually help in addressing first feeding, mortality, malformation, nutritional needs and environmental requirements [18–20].

Morphological characterization has been considered as an integral aspect that combines ecology with functional divergence [21]. Geometric morphometric tool has been effectively used for the quantitative statistical description of the organism's shape. Only limited reviews are available on the empirical expression of dynamic transformation patterns during the post-embryonic development of marine fishes [12, 13, 17, 22–31]. Allometry, the covariation pattern between several morphological features or between size and shape measurements [32], can be used to describe the evolutionary developmental history of the animal's growth trajectories [33], which in turn contribute to morphological integration [34]. The allometric variation could make it easier to cover the whole shape variation and contribute to the overall shape integration [34–36]. The reef fish is considered as a better model for studying the evolution of phenotypic plasticity, mainly when categorizing allometric and non-allometric patterns of growth [23].

Dascyllus carneus Fischer, 1885 (cloudy damsel) was successfully propagated and reared at the ICAR-Vizhinjam Regional Center of CMFRI, Kerala, India [37]. In the current study we explored the allometric and non-allometric growth pattern with stage-specific morpho-functional development and integration (head and trunk) of a poorly studied ornamental reef fish, *D. carneus* from yolk sac to juvenile stage.

Result And Discussion

Stage-specific size and shape variations: Phenotypic plasticity

Procrustes ANOVA analysis (Table 1) unveiled that each developmental phase has its unique shape and size, showing significant phenotypic plasticity ($p < 0.001$). Highest heterogeneous (phenotypic plasticity) group among this was DCTS, followed by DCPF, DCPS, DCYS, DCFS, and DCJS in the same order. Error MS value was found lower than individual MS value, so the data sets were devoid of any land marking error. Based on the analysis, DCJS can be concluded to be a stable stage. All others showed a highly fluctuating developmental phase with high degrees of phenotypic plasticity, particularly in shape and size, as a function of ontogenic development. The phenotypic plasticity of a population largely depends on genetics and gradients in the environment, which may also lead to changes in the ontogenic trajectories [38, 39].

Table.1: Stage-specific size and shape phenotypic plasticity. DCYS – Yolk sac stage; DCPF – Pre-flexion stage; DCFS – Flexion stage; DCPS – Post-flexion stage; DCTS – Transition stage; DCJS – Juvenile stage.

Sl. No.	Stage	Parameter	Effect	SS	MS	Df	F	P
1	DCYS (n = 42)	Centroid size	Individual	0.911628	0.045581	20	11.68	< 0.0001
			Error	0.081985	0.003904			
		Shape	Individual	0.06089453	0.0001522363	400	10.37	< 0.0001
			Error	0.00616788	0.0000146854			
2	DCPF (n = 62)	Centroid size	Individual	9.105070	0.303502	30	243.32	< 0.0001
			Error	0.038667	0.001247			
		Shape	Individual	0.26938746	0.0004489791	600	22.60	< 0.0001
			Error	0.01231764	0.0000198672			
3	DCFS (n = 20)	Centroid size	Individual	0.217523	0.024169	9	5.71	0.0059
			Error	0.042388	0.004239			
		Shape	Individual	0.03228958	0.0001281332	252	6.616	< 0.0001
			Error	0.00582828	0.0000208153			
4	DCPS (n = 26)	Centroid size	Individual	5.749079	0.479090	12	63.12	< 0.0001
			Error	0.098678	0.007591			
		Shape	Individual	0.03810840	0.0001134179	336	3.42	< 0.0001
			Error	0.01205819	0.0000331269			
5	DCTS (n = 24)	Centroid size	Individual	38.645122	3.513193	11	333.60	< 0.0001
			Error	0.126373	0.010531			
		Shape	Individual	0.03586379	0.0001164409	308	2.82	< 0.0001
			Error	0.01387326	0.0000412895			
6	DCJS (n = 86)	Centroid size	Individual	2013.233862	47.934140	42	3.39	< 0.0001
			Error	608.561810	14.152600			
*SS-Sum of squares, MS-Mean Square, Df-Degree of freedom, F-F-statistics, P-Probability								

Sl. No.	Stage	Parameter	Effect	SS	MS	Df	F	P
		Shape	Individual	0.15451937	0.0001313940	1176	6.29	< 0.0001
			Error	0.02514642	0.0000208857			
*SS-Sum of squares, MS-Mean Square, Df-Degree of freedom, F-F-statistics, P-Probability								

The morphological development and growth patterns during ontogeny closely match the immediate needs of the larvae^[17]. Regression analysis revealed that *D. carneus* did not change their linear pattern of allometric growth trajectories (Fig. 1D) up to DCPS stage. In contrast, non-allometric growth analysis indicated a sudden shift from negative to positive allometric growth after the DCPS stage (Fig. 1E). The cloudy damselfish followed positive allometric growth trajectories after the initial developmental stage (DCYS) in normal conditions. Negative allometric growth trajectories had been observed in common carp during early stages; the eyes grow quickly after hatching (for feeding functions- detection, approach, aiming, and capture), but later growth becomes positive allometric^[40]. The positive allometry between anterior and posterior parts of the body can be considered as a function to reduce the water resistance during swimming^[14, 41]. The energy usage during swimming is higher in larvae and it reduces as it advances in metamorphosis^[42, 43].

Yolk sac stage (DCYS) (0–3 dph)

In a total of 20 PC (allometric), the first three PCs revealed a total variation of 80.91%, which is contributed by PC1 (60.56%), PC2 (11.76%), and PC3 (8.58%) alone (Fig. 2A1). In the PC1, the highly variable landmarks were present in the caudal region (landmarks 10, 11, 7, 12). Furthermore, only slight variations were observed in the mouth and anal region (Fig. 2B1). Considering PC2, the highly irregular shape changes were observed, especially in the caudal and anterior regions (head region). However, landmarks 4 & 12 showed only slight variations compared to all other regions (Fig. 2C1). A different outcome was observed in PC3, where all the landmarks were displaced and showed irregular shape variations. The erratic movement of landmarks of the eye, reduction of the caudal region, and enlargement of the yolk sac area were the major characteristic features highlighted in PC3 (Fig. 2D1). Among 20 PCs present in the non-allometric analysis, the first three PCs covered the sum of 74.53% variance, which is solely constituted by PC1 (46.37 %), PC2 (16.24 %) and PC3 (11.92 %) (Fig. 2A2). The long and slender body, a major characteristic feature was observed in PC1. The landmarks 3, 8 and 1 were highly conserved in PC1 (Fig. 2B2). The PC2 (Fig. 2C2) and PC3 (Fig. 2D2) showed irregular shape and size variations, especially enlargement of basal body region and the posterior part of the head region.

In *D. carneus*, the yolk sac larvae were planktonic and free-floating just below the water surface with very weak swimming movements. No external feeding was observed at this stage. During this stage, the external fin fold undergoes resorption, yolk sac gets fully reabsorbed and the mouth opens synchronously [37]. Usually, larger eggs and yolk reserves slow down the developments in newly hatched larvae. In addition to that, lower oxygen diffusion rates (limiting the metabolism) during this stage restrict the expected growth pattern as in free-swimming stages [44, 45]. In *D. carneus*, eggs are comparatively smaller with very less yolk reserve. The DCYS stage last only for short period. The random and lower rate of morphometric variations in most of the landmarks at this stage might be a reflection of the presence of yolk sac and an underdeveloped respiratory system.

Preflexion stage (DCPF) (4–10 dph)

The allometric changes of this stage represent a total of 20 PCs; the first three PCs covered a total variation of 70.55%, which is contributed by PC1 (37.67%), PC2 (23.78%) and PC3 (9.10%) alone (Fig. 2A3). The PC1 highlighted the ocular changes (indicating active movements in the loci of the eye) and orientation of caudal regions (for pelagic lifestyle-round caudal fin). Around 37.67% of variations in *D. carneus* of the DCPF stage were characterized by the upward-oriented mouth (the lower jaw being longer than the upper jaw) (Fig. 2B3). This kind of development occurs in surface feeders [46]. Both PC2 (Fig. 2C3) and PC3, showed similar characteristic features like round caudal fin with a clear convex head. One of the striking features in the PC3 was the downward movement of landmark no. 9, which reflected the increment in the depth of the lower orbicular region at the lower jaw to support exogenous feeding (Fig. 2D3). The allometric and non-allometric growth patterns of the DCPF stage showed almost similar kind of shape and size variations. The first three PCs (non-allometric) covered a total variance of 72.32%, which were exclusively constituted by PC1 (38.97%), PC2 (24.61%) and PC3 (8.75%) (Fig. 2A4, B4, C4 & D4). Most of the morphometrical changes, including the development of caudal, dorsal, and anal fin anlagen, were observed during the DCPF stage, when the visual feeding starts. The preflexion stage is one of the most critical metamorphic steps and it extends from 4 to 10 dph. At this stage, the fish changes its planktonic lifestyle into free-swimming pelagic. They begin to swim actively and also feed on exogenous materials (live feeds) [41, 37]. After the eleuthero-embryonic stage, the larvae are characterized by the developments in the orobranchial region [47] as well as tail region, in tune with the developments in their feeding and swimming adaptations [16, 48]. During the preflexion stage the developments in the eye and nervous system enable the larvae to engage in visual detection of live feeds. The larvae react to light stimuli and prefer exogenous feed to cope up with the depletion of yolk reserves [49]. The shifting of cutaneous to branchial respiration results in increased oxygen supply and in turn helps the larvae to engage in active swimming activities. The modifications acquired in the feeding apparatus at this pelagic stage may also improve larval growth and survival [50–52]. The successful transition of a non-feeding yolk sac stage larva into an exogenous feeding stage is an important critical event that determines the survival of the larva.

Flexion stage (DCFS) (11–12 dph)

In PCA, out of 10 PCs, the first three PCs revealed a total variation of 77.45% which is contributed by PC1 (39.65%), PC2 (28.93%) and PC3 (8.88%) alone (Fig. 2A5). The PC1 showed limited variation compared to the average structure of DCFS. The backward movements of the dorsal and ventral fin with enlargement of the caudal fin are the major characteristic features observed in the PC1 (Fig. 2B5) and PC2 (Fig. 2C5). In *Pomacentrus amboinensis*, the analogues of the dorsal and anal fins are formed at this stage, along with pelvic fin buds ^[25]. In *D. carneus*, all three PCs showed slightly superior mouth (forward movement of landmark no. 9) (Fig. 2B5, C5, & D5). In the case of non-allometric analysis, the first three PCs of total nine PCs covered a total variance of 77.06%, which is solely constituted by PC1 (41.39%), PC2 (27.73%), and PC3 (7.94%) (Fig. 2A6). The allometric and non-allometric PCs covered almost similar shape and size features (Fig. 2B6, C6, & D6).

In DCFS, the larval growth was observed both lengthwise and depth-wise and the major changes occurred at the notochord end. It curves upward to attain a vertical position and this stage lasts only for a short period of two days. This flexion process influences many anatomical, hormonal and morphometric changes. The alimentary canal becomes fully functional with the development of internal digestive and other accessory structures. Muscular development and caudal fin formation enabled the larvae to acquire active swimming capabilities ^[2,14, 17, 41, 47 53– 56].

Postflexion stage (DCPS) (13–15 dph): In allometric characterization, the major variations accounted in 13 PCs; the first three PCs revealed a total variation of 64.49%, which is contributed by PC1 (31.05%), PC2 (20.62%) and PC3 (12.82%) alone (Fig. 2A7). However, in the case of non-allometric components, the first three PCs contained a total variance of 66.14%, which is exclusively constituted by PC1 (30.80%), PC2 (21.93%) and PC3 (13.42%) (Fig. 2A8). The primary features of both allometric and non-allometric analysis in PC1 include enlargement of the eye, convex dorsal head region, well developed dorsal, ventral and caudal fins (Fig. 2B7 & B8). The backward movement of the dorsal fin is the predominant feature found in PC2 (Fig. 2C7 & C8) and a slender body with constricted dorsal and ventral fins was observed in PC3 (Fig. 2D7 & D8).

The major characteristic changes of DCPS occurred in the trunk and caudal region. The head region exhibited a gradual increase in size in conjunction with the development of the gill chamber, buccal cavity, and associated structures. The eye development gets stabilized by this stage ^[57]. Caudal peduncle gets strengthened and the length of the trunk increases, which leads to the strengthening of muscles to attain balance in swimming, feeding and for better escape reflex ^[16, 48]. In case of a similar species, *Pomacentrus amboinensis* at postflexion larvae, Murphy *et al.*, (2007) an increase in the linear measurements and 32–39% increase in head size was observed ^[25]. The blunt snout was observed to become concave and less oblique and development of fins was completed. The fin structures got ossified to support the overall development in size and the activities of the fish larvae during this stage ^[2, 17, 24, 41, 47, 54–56].

Transition stage (DCTS) (16–25 dph): In the allometric (12 PCs) (Fig. 2A9) and non-allometric (11 PCs) (Fig. 2A10) characterization, the PC1 (55.43% in allometric & 58.91% in non-allometric) of both analyses did not show significant ($p < 0.1980$) variations in shape and size. The major features were a forward movement of the dorsal fin and reduction of the head size (Fig. 2B9 & B10). But in PC2 (allometric-17.04%

& non-allometric 15.35%) (Fig. 2C9 & C10) and PC3 (allometric 12.41% & non-allometric 11.05%) (Fig. 2D9 & D10) of allometric and non-allometric analysis, most of the features, except the size (non-allometric analysis has shown slightly expanded body), were identical,

Asynchronous growth pattern was observed in DCTS and major changes occurred in the trunk region. Growth of head and caudal regions got stabilized. At this stage the larvae begin to settle at the bottom, squamation process is initiated and sensory structures begin to develop [2, 17, 41, 47, 54–56]. Escape reflex, predatory and territorial behaviors were evident at this stage. Dorsal, anal and caudal fins were fully developed and ossification continued. As the process of squamation and muscle development progressed, the fish becomes opaque [25]. Pelvic fin anlage was visible at this stage. At the end of this stage, the pigmentation pattern of the larvae also changed.

Juvenile stage (DCJS) (26–55 dph): A total of 12 and 11 PCs were present in the allometric (Fig. 2A11) and non-allometric (Fig. 2A12) analysis respectively. Both the analyses showed similar features except in the case of landmark no. 5 (dorsal edge of the head perpendicular to the eye). The variations in this region determined the dorsal head profile of *D. carneus* i.e., clear convex profile in front of the dorsal fin [58]. Allometric analysis of the first three PCs revealed a total variation of 77.98%, which is contributed by PC1 (57.82%) PC2 (14.74%) and PC3 (5.33%) alone (Fig. 2B11, C11 & D11). In the non-allometric analysis, the first three PCs covered a total variance of 77.38%, which were solely constituted by PC1 (57.24%), PC2 (14.64%) and PC3 (5.51%) (Fig. 2B12, C12 & D12).

During the DCJS, the larvae completed their metamorphosis. The shape of the fish changed to compensate for the water-resistance during swimming [41, 59] and to accommodate the presence of well-developed dorsal, caudal, anal and pelvic fins. The colouration simulates the adult, the fish occupy a specific territory and aggressive interactions are observed between individuals [16, 48]. The mouth became more prominent and was positioned more towards the anterior terminal edge of the head. Even though the terminal mouth orientation has been one of the most common characteristic features of the genus *Dascyllus*, in *D. carneus*, the mouth was seen to be slightly superior.

Interestingly, the allometric as well as non-allometric analysis, showed the same variations after the DCYS stage. Environmental variables strongly influence the outcome of the genetic makeup of an organism and morphological difference within the species. Gene expression is seen to be tightly regulated and in tune with the combinations of environmental factors such as salinity, temperature, depth of the water column, dissolved oxygen, current flow effect, food availability, foraging behaviour, and habitat use [22, 27, 60–66]. In addition to the environmental factors, population density and fishing mortality can also influence growth and survival [67–69]. Each of these variables can have significant functional, eco-morphological implications for locomotion, visual acuity, prey handling and microhabitat use [70, 71]. Each coral reef fish has unique ontogenetic trajectories, especially in the case of *Dascyllus* spp., the interspecific divergence of adult morphology is the result of the differences in the duration of developmental stages, ontogenetic trajectories or lateral shifts of the trajectories and changes in the size-shape space [26]. From a functional point of view,

in the cloudy damselfish, the gradual process of post-embryonic development (yolk sac to juvenile) results in a well-adapted or fittest homogenous population (i.e. DCJS) [29, 72, 73].

Discriminant function analysis (DFA)

DFA is used here for testing the hypotheses of morphological similarity or dissimilarity between the developmental stages. Here, we evaluated the shape transformation between the adjacent developmental stages (Fig. 3).

The allometric and non-allometric analysis of PCs showed that, all post-embryonic development stages in *D. carneus*, except the DCYS stage, followed allometric growth patterns (Fig. 1D). Most of the variations occurred in landmark no. 5, dorsal and ventral fin, as well as in caudal regions. DFA revealed that DCYS and DCPF stages exhibit significant variations between them and most of the variations were observed around the mouth region (initiation of feeding), eye, and dorsal head. Development of the eye ensures prey capture success. Moreover, this indicates the development from a non-feeding to feeding stage^[41]. Landmark 2, L (center of the eye) remains in the same loci; while landmark 5 (dorsal edge of the head) (l) shifts vertically upward and landmark 9 (lower edge of the head) moved vertically downward. These movements in the positions of landmarks resulted in the deepening of the head region. The secondary cephalization facilitated the development of head to accommodate the expansion of gill chambers. At this stage, the trunk area showed the highest variation among all other loci. This is because of the successive development of muscles and visceral content^[47, 55, 56]. The tip of the notochord, i.e., landmark 7 and the tip of the tail, i.e., landmark 12 remain almost the same during this segment of development. In conclusion, the variation from the yolk sac stage to the preflexion stage mainly represents the development in the head and trunk regions along with a slight variation in the caudal region.

DFA of DCPF and DCFS stages (Fig. 3A2 & B2) were more dynamic and represented a major part in the developmental process since it included the flexion process. The further anterior shifting of landmark 1 denotes the changes in the feeding behavior. At this stage, the enlargement of the buccal cavity and development of active feeding were observed^[47, 55, 56]. Landmarks 2, 3 and 4, represent the eye center and eye diameter respectively, showing a lesser rate of variation at this stage, which indicates that the eye development was a primary function in the ontogeny which has already been completed during the development from yolk sac to the preflexion stage itself^[40]. Furthermore, this depicts the elongation of the digestive tract, muscular development, swimming activities, better feeding, escape reflex and territorial behavior based on the adaptations in the larval morphology^[2, 17, 41, 47, 54–56]. The landmarks 10 and 11 moved apart from each other as the caudal peduncle became functional and larvae acquired additional swimming abilities. In turn, it supported the flexion process, where the landmark 7 moved upward to indicate the flexion of the notochord. Synchronously, the caudal fin showed extensive development which shifted the landmark 12 to the longitudinal plane of the larval body and tip of the tail. In conclusion, the variation between the preflexion and flexion stages was represented by the lengthening of the trunk and tail region, development of caudal peduncle and flexion of notochord.

DFA of DCFS and DCPS stages (Fig. 3A3 & B3) were marked by limited changes compared to the previous stages. At this segment of development, the head region became less dynamic in growth. The trunk region

is designated with four new landmarks representing dorsal and anal fin anlagen. The landmarks 6 and 8 slightly moved apart which depict deepening of the trunk to accommodate the increased mass in the abdominal region and development of muscle structures^[74, 75]. Moreover, the movements of landmarks in the trunk (landmarks 14 & 16) towards the caudal peduncle reflected the growth of larvae in length. As the landmarks 10 and 11 moved apart the caudal peduncle width visibly increased and this could be attributed to increased swimming ability. The caudal fin principally maintains the body balance during swimming, while the trunk region continuously grows both in length and width^[2, 17, 41, 54, 55]. The flexion process is completed as landmark 7 moved completely vertical to the body axis. Changes in landmark 12 represented the dynamic developments of the tail. It slightly moved backward as a result of the growth of larvae and aligned more towards the central longitudinal plane of the larval body. In conclusion, during this development segment, the head region remained comparatively the same, but the trunk and tail region encompassed major variations. Besides, the dorsal and anal fin anlagen also appeared.

Figure 3A4 & B4 represents the DFA of DCPS and DCTS stages, which are the quintessential segments of ontogeny; probably by this stage larvae acquire more functional adaptations to become stable as an individual. Well-developed eyes (landmarks of eyes well fixed) might support the overall growth of fish and enable the fish to have better visual abilities. Enlargement of head regions (landmark 5 & 9) was observed to accommodate the developments in the buccal cavity, gills, and brain^[17, 54, 55]. Landmark 5, 6, 8, and 9 remained in line with each other indicating synchronous growth in the trunk region. In conclusion, at this stage most of the developments were internal and it is the preparatory phase for pre-metamorphosis.

DFA of DCTS and DCJS stages (Fig. 3A5 & B5) revealed little or no change to the head, trunk and tail regions. The snout was seen equally situated between landmarks 5 and 9 and form the terminal mouth at this stage. The eye dimensions remained the same. Slight changes were noticed at landmarks 13 and 6, as these loci further moved down and the dorsal part acquired a more oval shape. This in turn helped the fish to reduce the water resistance during swimming. Landmark 15 moved and established in line with landmark 8. The tip of the tail represented by landmark 12 moved further back, indicating the increment in the caudal fin length and total length. It can be concluded that, in this developmental segment, the body plan became compact and streamlined according to the dynamics of the living medium. The caudal region remains unchanged, but the caudal fin expanded and became fully functional.

These stages prepared the larvae for metamorphosis. Within the population, differential growth may occur which in turn affects the period of metamorphosis. Post embryonic developmental events in *D. carneus* like scale formation, body coloration, fin and fin rays formation were completed in this segment of development. Metamorphosis of *D. carneus* is synchronous with the behavioral interactions (both inter and intraspecies) such as competition for feed, territory and companionship. During the transition stage, squamation gets completed, which pave the base for colour change during metamorphosis.

Metamorphosed juveniles were marked with unprecedented changes in its colour on par with adults^[37].

Successful breeding and larval rearing practices require comprehensive scientific knowledge about the vital stages such as hatching, initial feeding, mortality, malformation, and nutritional and environmental requirements. Mortality of the larvae coincide with the major ontogenetic events such as flexion initiation

(preflexion), flexion stage, transition stage and final metamorphosis^[18–20]. Comparison of growth levels in developmental stages and corresponding changes in the life-history may explain the interdependency between them^[58]. From a functional point of view, the comparative analysis of stages with successive stages quantifies the changes that occurred over time. The morphological changes play a key role in the survival, adaptations, and choice of prey, which in turn can lead to changes in the ability to forage and subsequently the exploration of food resources^[74, 75]. Quantitative ontogenetic data, which accounts for individual variations give access to a more profound understanding of the developmental process^[76, 77]. The geometric morphometric (GMM) system has already been proven effective in identifying stocks and improving the biological basis of fish stock management^[78]. This is used here to explain the ontogenetic variations. Analysis of morpho-functional characteristics of a species is essential to understand the dynamics of the population and the factors affecting them at different developmental stages^[79, 80]. Knowledge about morpho-functional ontogeny can be effectively used in hatchery practices by identifying species-specific needs^[81].

Developmental integration of head and trunk

Integration of head and trunk within the configuration of landmarks was examined through Partial Least Square (PLS) analysis. In DCYS, PLS1 represented 89.98% of the total variance and the RV coefficient was 0.663 ($p < 0.001$ at 1000 permutational analysis). The RV coefficient was higher than 0.5 (null hypothesis was rejected), which indicated strong covariation between the blocks^[82, 83]. This in turn establishes the strong synchronous development pattern of the head and trunk. Any structural and functional changes that have arisen in the head or trunk region will surely alter the shape and size of the associated regions (head or trunk and vice versa).

Critical stages in the development of cloudy damselfish are DCYS (Fig. 4A) and DCPF (Fig. 4B) (RV coefficient is 0.940, $p < 0.001$ which covered the 98.47% of total variance), and both showed a high degree of compactness in the head and trunk regions. From the DCFS stage, the covariation between the blocks shifted considerably. In the DCFS, PLS1 covered 62.65% of the total variance with an RV coefficient of 0.192 ($p = 0.158$). Here, the RV coefficient value was lower than 0.5, indicating that the variation between the blocks was weak, but within blocks, this interaction remained strong. However, the RV coefficient in DCFS was less than 0.5, indicating no substantial association between the blocks (Fig. 4C). Nevertheless, the increase in RV coefficient is ideally within the boundary of 0.5 or below - DCPS (RV coefficient 0.522, $p < 0.001$ with covering 87.82% of total variance) (Fig. 4D), DCTS (RV coefficient 0.469, $p = 0.001$ with covering 75.80% of total variance) (Fig. 4E) and DCJS (RV coefficient 0.505, $p < 0.001$ with covering 82.97% of total variance) (Fig. 4F). Based on this study, we can conclude that DCYS and DCPS growth occurred as a single whole unit (head and trunk). The increase in functional modularity was observed from the DCFS stage and the ideal modularity or PLS block of covariation was observed in the DCTS stage.

There is a significant challenge in determining the decoupling process (dissociation of blocks)^[84] from the DCFS stage while the coupling and decoupling processes during morphogenesis in the head and trunk of cloudy damselfish seem to have an impact on their evolutionary constraints. The covariation pattern may contribute to the extensive morphological adaptation, survival and fitness of the organism^[85, 86]. Though

few reports focused on the development of neurocranium and mandible processes [26, 28, 29], this study forms the first report on the complete ontogenetic covariation of a reef fish. The ontogenetic allometry of the neurocranium and mandible of selected *Dascyllus* species is well explained [28]. A fairly similar mandible type for each species of *Dascyllus* is probably related to their diet, which composed essentially of copepods [87]. Although neurocranium is a structural unit with various functions, it is also influenced by the size of eyes, brain structure and feeding [88, 89] and swimming performances [90]. Furthermore, these complexities of demands as a whole contributed to the shape and variability in the neurocranium of giant (*D. trimaculatus* & *D. flavicaudus*) and small-bodied (*D. aruanus* & *D. carneus*) species. The vertebrate crania are highly integrated both genetically and functionally [91–93] while, the disparity in shape of neurocranium and the mandible demonstrated a modularity function [94]. In our study, the head and trunk are in fairly separate morphology module after the initial developmental stages (DCYS and DCPF). The same strategy is visible in the allometric evolutionary pattern of the neurocranium and the mandible shaping of *Dascyllus trimaculatus* and *Dascyllus flavicaudus* [28].

Shape and size similarities between the *D. carneus* metamorphic stages

Two block-Partial Least Square (PLS) (Fig. 5) interpretations were performed to examine the relationship between the shape (Block 1) and size (Block 2) of the adjacent developmental stages of *D. carneus*. First, we quantified the relationship of shape between all development groups (12 landmarks based). The study concluded that DCFS and DCPS had significantly very high similarity ($p < 0.0001$) of shape relative to other groups. The RV coefficient was 0.398 using 10000 permutation analyzes covering 100% of the total variance (Fig. 5A2). Nevertheless, in the centroid size analysis of these developing stages, the DCJS stage showed well-noted changes in size compared to other stages and the DCTS stage showed similarity in size with DCJS and DCPS (Fig. 5B2). This analysis confirmed that each stage of development has its unique shape and size features. Furthermore, we also tried to measure the influence of newly formed regions on the shape and size of ontogenetic trajectories in 16 landmark-based groups. The development of additional loci (landmarks 13, 14, 15 & 16) had a significant effect on the shape and size of post-embryonic development. Among the four phases (DCFS, DCPS, DCTS & DCJS), DCJS and DCPS showed unique shape features compared to the other two phases (Fig. 5A1). In the size analysis, all phases showed unique properties and also shared some basic features (Fig. 5B1). Phenotypical changes associated with body size variations during the development are covered in the ontogenetic allometry, which helps to explain how different factors respond to phenotypes during the different stages of development [35]. However studies indicated that the dynamics of ontogenetic development are likely to be highly regulated by phenotypical selection forces [95]. Therefore, the analysis of allometric trends across the species has been an essential approach to identify the characters that contribute to phenotypic variations during ontogenetic development [35, 96].

Canonical variate analysis (CVA)

CVA analysis (Fig. 5A3 & B3) with 12 landmarks covering all six stages has indicated the connectivity of the neighboring stages and the differences between the distant stages. DCYS resembles the DCPF stage but showed a clear distinction from the other stages. DCFS and DCPS stages showed closeness but varied

from all other forerunner and sequential stages. The stages, DCTS and DCJS were similar but both were different from all other forerunning stages. CV1 alone showed a variation of 66.89% and CV2 had a variation of 23.26% and these together form a variation of 90.15% (significant at $p < 0.0001$ in 10000 permutational analysis) (Fig. 5B3). In 16 landmarks-based-CVA which includes 4 developmental stages (DCFS to DCJS), all stages exhibited a unique morphospace, nevertheless leaving some degree of influence or similarity between the stages. Among all four phases, similarity existed between DCFS-DCPS and DCTS-DCJS. The CV1 covered 81.55% of the total variance, and CV2 covered 12.04% of variance and these were significant at $p < 0.0001$, in 10000 permutational analysis (Fig. 5A3). Overlapping and separation of body shape in morphospace could be due to genetic, allometric trajectories, evolutionary adaptations, and interactions with the surrounding habitat. The selection pressure associated with the surrounding environment contributed to the phenotypic plasticity of the fish in response to biophysical factors (food, temperature, pH, water current, inter and intraspecific competitions, etc.), by altering their morphology and behavior, which eventually led to changes in their morphology, reproduction, and survival ^[78]. Natural selection may foster phenotypical divergence and also increase their functional adaptations related to their surrounding habitat ^[97–100].

Association between the size and shape of each stage

Interestingly, significant size-related shape changes occurred in the alternative developmental phases of *D. carneus*, such as DCYS ($p < 0.0001$ with covering 32.61% of allometric residue), DCFS ($p = 0.01$ with covering 14.37% of allometric residue), and DCJS ($p < 0.028$, predicted allometric residue 3.73%). The other stages such as DCPF ($p = 0.07$ with covering 3.35% of allometric residue), DCPS ($p = 0.12$ with covering 6.37% of allometric residue), and DCTS ($p = 0.198$ with covering 6.4% of allometric residue) did not show any significant ($p > 0.05$) correlation between the size and shape. Due to the absence of size-related shape changes, during this segment of development, it may be concluded that the major changes were restricted to the development of anatomical structures and their organization. Generally, ontogenetic shape differences would allow higher species packing, even if juveniles of one species and adult of another had the same shape, they would still vary in size, allowing ecological stratification ^[101]. The evidences of actual relation between survival, larval size, and condition of a population are scanty, but nevertheless larval size and biochemical compositions are two variables that potentially alter mortality trends ^[102]. Therefore, these features of individual larvae may be used to support the hypotheses about the possible susceptibility of larval cohorts to starvation and predation stress ^[103–105]. Investigation on the importance of size for survival during the early stage of life-history has provided mixed results ^[106, 107]. There is evidence to suggest that larger larvae at a particular stage of development have advantages over small larvae due to greater access to a wide range of food sizes, enhanced competitive functions, swimming abilities, and superior escape reflex ^[108]. Contrary to conventional thinking, larger larvae experience a higher level of predation pressure ^[109].

A common concept in evolutionary developmental biology is that phenotypic variability results from early developmental changes, which are designed to create diverse morphologies for adults ^[84]. However, our findings suggest that phenotypic plasticity at the early stages of development is compensated or counteracted by the DCTS stage, which in turn lead to the development of highly conserved species-specific

phenotypes (*i.e.*, DCJS). It is interesting to note that, the aggressive nature of *Dascyllus* spp. in their social systems, did not play a major role in the distribution of juvenile; and also not in the survival and mortality of an organism under wild conditions^[30, 110–112]. This suggests that natural selection can have a positive effect in selecting those changes in the ontogenetic trajectories that lead to new morphological forms, which in turn reduce the intraspecific competitions^[31]. In the selection process, the DCTS is one of the crucial checkpoints for the survival of organisms where a substantial number of variations minimize intraspecific competitions in the wild^[113]. However, in our study, each developmental stage showed considerable difference in ontogenetic trajectories and head-trunk covariations. In *D. carneus* also higher levels of aggression and mortality were observed between DCFS and DCTS developmental stages^[37]. The hatchery reared larvae have been observed to be substantially different from the wild population with respect to nutritional profile, muscle development, growth rates^[114] and swimming skills^[115]. Many external factors (food supply, temperature, pH, water current, salinity, etc.) and internal conditions (physiology, sex, behavior, etc.) may also be responsible for individual variations in shape^[26], which require further study. Finally, this information about shape may be used to complement traditional growth curves, showing how and when shape changes occur^[66]. Our findings shed some light on a poorly understood part of the biological growth trajectory studies. This type of mathematical modelling and explanations of stage-specific morpho-functional phenotypic plasticity and constraints would aid in better understanding of the ontogenetic developments and also help in delineating the critical stages in larval rearing. The knowledge would help to understand the adaptations of the larvae to environmental conditions and survival needs, which are the prime focus in the hatchery, where standardization of larval survival is a major bottleneck for reef fishes.

Methods

Ethics statement

All the *in vitro* protocols and procedures involving fish were performed as per the the licence No. 08 – 1/20 granted to the Project Personnel under the Institutional Animal Ethics Committee of ICAR-Central Marine Fisheries Research Institute, Government of India, Kochi (613/GO/RBi/S/02/CPCSEA) and also with the written approval by the Local Ethical Committee at Vizhinjam Regional Centre of ICAR Central Marine Fisheries Research Institute, Vizhinjam. The studies were under-taken at ICAR-Vizhinjam Regional Centre of Central Marine Fisheries Research Institute, Vizhinjam, Thiruvananthapuram, compliance with the general guidelines of local and global ethical policies of Government of India.

The entire experimental protocols and procedures involving fish were performed in compliance with the ARRIVE guidelines.

Test species and procedure

A total of 260 number of larvae of *D. carneus* were used for the entire study. The larvae were anesthetized using MS222 before the study procedures^[116, 117]. Different larval stages and juveniles stages were photographed appropriately. Yolk-sac stage was photographed using Zeiss Axio Lab.A1 compound

microscope attached with ZEISS Axiocam ERc 5s with ZEN 2 lite software. Successive larval stages were photographed using Leica S8APO stereo zoom microscope connected to camera (Leica DFC 290) with a software Leica application suite version 4.1.0. Late larval stages and juveniles were photographed using digital cameras (Nikon D7100 & Nikon D90). The photographs of the specimens were staged into yolk-sac (DCYS), pre-flexion (DCPF), flexion (DCFS), post-flexion (DCPS), transition (DCTS) and juvenile stages (DCJS) ^[37]. Based on morphological taxonomy, a total of 12 homologous landmarks were identified in DCYS and DCPF stage and 16 landmarks were identified in the DCFS, DCPS, DCTS & DCJS stages (Fig. 1A, B & C). In order to check the error in the landmarking procedure, individual samples were digitalized twice. A Procrustes ANOVA was conducted to determine whether the mean squares (MS) for the samples were lower than the error ^[34]. Drawings were done using CorelDRAW Graphics Suite X5.

Geometric morphometric (GMM) analysis

2D-images of the post-embryonic developmental stages of *D. carneus* (DCYS, n = 42; DCPF, n = 62; DCFS, n = 20; DCPS, n = 26; DCTS, n = 24; & DCJS, n = 86) have been converted to TPS file format by using tpsUtil V1.68 and specimens were landmarked using tpsDIG V2.26 ^[118]. The analysis of allometric and non-allometric shape and size variation of each developmental stage was carried out ^[34, 119]. All geometric morphometric analyses were performed in the MorphoJ tool ^[120].

Discriminant function analysis (DFA)

DFA was used to discriminate the shape and size variations of the adjacent developmental stages ^[121, 122]. Five different bordering combinations like YS-PF, PF-FS, FS-PS, PS-TS, and TS-JS were selected.

Developmental association of head and trunk: PLS (Partial least square) analysis within the configuration was used to analyze the degree of association between the head and trunk of each developmental stage of *D. carneus*. This analysis identifies the difference in shape that most closely covariates between blocks and indicates their relative contribution to the overall covariation between blocks ^[123, 124, 83]. For the morphometric study of variation, the emphasis was on the two configuration landmarks (head and trunk), in 12 landmark stages, Block 1 (head : landmarks 1,2,3,4,5,9) and Block 2 (trunk : landmarks 6,7,8,10,11,12) and in the case of 16 landmarks developmental stage Block 1 (head : landmarks 1,2,3,4,5,9) and Block 2 (trunk: landmarks 6,7,8,10,11,12,13,14,15,16).

Shape and size relationship of developmental stages

Evaluation of the similarity of shape and size of developmental phases of *D. carneus* was accounted for using two-block PLS analysis ^[123]. In this analysis, we quantified the shape and size of two separate groups, based on 12 and 16 landmarks. The size-related shape changes of each development stage were monitored by means of regression analysis and the size was defined as independent variable and shape as dependent variable with 10000 permutations. Multivariate regression analysis was done to evaluate the association between the size and shape ^[34].

Canonical variate analysis (CVA)

CVA is a method used to identify shape characteristics distinguished between multiple groups of specimens ^[125, 126]. The CVA finds shape values that optimize values within the group variations, assuming the covariate matrix is similar ^[127]. Here, a total of six different developmental stages were used for morphometric analysis. The first two stages had 12 landmarks and the rest had 16 landmarks. Initially, CVA was performed with all groups having 12 landmarks (considered as a single group), while the stages having 16 landmarks were analyzed separately. Each analysis was performed using 10000 permutations.

Declarations

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Author contributions

F M A-Data collection, data interpretation, drawings, plate preparation and manuscript preparation

B S-Manuscript preparation

P P A- Data analysis, Data interpretation and manuscript preparation

Y S V-Data analysis and plate preparation

A G -Manuscript preparation

P K- Manuscript preparation

Additional information

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Figures

Figure 1

(A) *Dascyllus carneus* – Adult (B) *D. carneus*– pre-juvenile stage with landmarks (C) Landmarks descriptions (D) Allometric regression growth trajectories (E) Non-allometric regression growth trajectories.

YS – Yolk sac stage; PF – Preflexion stage; FS – Flexion stage; PS - Postflexion stage; TS – Transition stage; JS – Juvenile stage.

Figure 2

PCA of all developmental stages of *D. carneus*, (A) Percentage of variance (B) PC1 (C) PC2 (D) PC3. The odd numbers indicate the allometric PC analysis and even numbers indicate the non-allometric PCs of each developmental stage. YS – Yolk sac stage; PF – Preflexion stage; FS – Flexion stage; PS - Postflexion stage; TS – Transition stage; JS – Juvenile stage.

Figure 3

Discriminant functional analysis (DFA) of adjacent developmental stages. (A1-A5- DFA graph of adjacent development stages), (B1-B5-Wireframe graph of adjacent development stages). YS – Yolk sac stage; PF – Preflexion stage; FS – Flexion stage; PS - Postflexion stage; TS – Transition stage; JS – Juvenile stage.

Figure 4

Partial least square (PLS) – Covariation analysis of developmental stages. (A) Yolk sac stage (B) Pre-flexion stage (C) Flexion stage (D) Postflexion stage (E) Transition stage (F) Juvenile stage. Each covariation block is represented as each color dot code, Red dot for the head and cyan dot for trunk region covariation block.

Figure 5

Partial least square analysis (PLS) (A1) 16 landmarks-based shape analysis (B1) 16 landmarks-based centroid size analysis (A2) 12 landmarks-based shape analysis (B2) 12- landmarks-based centroid size analysis. Canonical covariate analysis (CVA), (A3) 16 landmarks based CVA (B3) 12 landmarks based CVA. YS – Yolk sac stage; PF – Preflexion stage; FS – Flexion stage; PS - Postflexion stage; TS – Transition stage; JS – Juvenile stage.