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RESEARCH ARTICLE ARAŞTIRMA MAKALESİ

Quantitative assessment of gula pigmentation in *Chironomus riparius* (Diptera: Chironomidae)

Chironomus riparius (Diptera: Chironomidae)'ta gula pigmentasyonunun nicel değerlendirilmesi

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Abstract: Chironomids (Diptera: Chironomidae) are among the most abundant and ecologically significant invertebrates in freshwater ecosystems. Within this group, the genus *Chironomus* poses considerable taxonomic challenges due to its high number of cryptic species, especially at the larval stage. Larval identification traditionally relies on qualitative morphological characters, such as the pigmentation of the gula. However, the subjective nature of these observations limits reproducibility and diagnostic accuracy. This study provides a quantitative assessment of gula pigmentation variation among *Chironomus riparius* larvae derived from a single egg mass. Image processing techniques were applied to convert color traits into measurable parameters, enabling objective analysis of pigmentation intensity, distribution, and optical properties. Digital quantification revealed significant intraspecific variation in gula pigmentation, even among genetically identical larvae reared under uniform laboratory conditions. These results underscore the potential of digital image analysis to improve the precision, objectivity, and reproducibility of morphological identification in chironomids. This methodological framework contributes to the standardization of qualitative traits for broader use in ecological and systematic research.

Keywords: *Chironomus riparius*, gula pigmentation, image analysis, morphometry, non-biting midges, phenotypic variation

Öz: Chironomidler (Diptera: Chironomidae), tatlı su ekosistemlerinde en bol bulunan ve ekolojik açıdan en önemli omurgasız gruplarından biridir. Bu grup içerisinde yer alan *Chironomus* cinsi, özellikle larva evresinde çok sayıda kriptik tür içermesi nedeniyle önemli taksonomik güçlükler barındırmaktadır. Larvaların tanımlanması geleneksel olarak gula pigmentasyonu gibi nitel morfolojik karakterlere dayanmaktadır. Ancak bu tür gözlemlerin öznel yapısı, tekrarlanabilirliği ve tanısal doğruluğu sınırlandırmaktadır. Bu çalışmada, tek bir yumurta paketinden elde edilen *Chironomus riparius* larvalarında gula pigmentasyonundaki varyasyon nicel olarak değerlendirilmiştir. Renk özelliklerini ölçülebilir parametrelere dönüştürmek amacıyla görüntü işleme teknikleri uygulanmış; böylece pigmentasyon yoğunluğu, dağılımı ve optik özelliklerinin nesnel biçimde analiz edilmesi sağlanmıştır. Sayısal analizler, genetik olarak özdeş ve kontrollü laboratuvar koşullarında yetiştirilen larvalar arasında dahi gula pigmentasyonunda anlamlı tür içi varyasyon bulunduğunu ortaya koymuştur. Elde edilen bulgular, dijital görüntü analizinin chironomidlerde morfolojik tanımlamanın doğruluğunu, nesnelliğini ve tekrarlanabilirliğini artırma potansiyelini vurgulamaktadır. Sunulan bu metodolojik yaklaşım, nitel karakterlerin standartlaştırılmasına katkı sağlayarak ekolojik ve sistematik çalışmalarda daha geniş kullanım olanağı sunmaktadır.

Anahtar kelimeler: *Chironomus riparius*, gula pigmentasyonu, görüntü analizi, morfometri, sokmayan sinekler, fenotipik varyasyon

INTRODUCTION

Chironomids (Diptera: Chironomidae), commonly referred to as non-biting midges, constitute one of the most ecologically dominant and diverse groups of aquatic invertebrates. They occur in nearly all freshwater habitats and often represent the largest proportion of benthic biomass (Karima, 2021). Functionally, chironomids play a key role in organic matter decomposition and nutrient cycling, thereby contributing substantially to freshwater ecosystem processes (Leszczyńska et al., 2019). Their wide ecological tolerance and species-specific responses to environmental change also make them valuable bioindicators in ecological and water-quality assessments (Nicacio and Juen, 2015). The genus *Chironomus*, which gives its name to the family, is particularly important both ecologically and taxonomically. With approximately 650 described species (Panis et al., 1994), *Chironomus* includes many cryptic taxa that are morphologically similar, especially at the larval stage. Consequently, larval identification often remains uncertain, and many specimens are reported collectively as *Chironomus* spp. Conventional identification relies heavily on external morphological traits such as the shape of the ventromental plates, antennal proportions, and the structure of the mentum and mandibles (Johannsen, 1937; Shobanov et al., 1996; Brooks et al., 2007). Among approximately twenty morphometric parameters typically used to differentiate species (Webb and Scholl, 1985), the gula—situated on the ventral head capsule—has long been recognized as an informative diagnostic feature. Gula pigmentation, in particular, has proven useful for distinguishing closely related *Chironomus* species, including *C. luridus*, *C. pseudothummi*, *C. melanotus*, *C. melanescens*, *C. piger*, and *C. riparius* (Kiknadze et al., 1991; Vallenduuk and Moller Pillot, 1997). However, descriptions of this character are traditionally qualitative—phrased in subjective terms such as "lightly pigmented" or "dark brown"—which reduces comparability and reproducibility among studies. The extent of natural variation in gula pigmentation within a single population, or even among larvae derived from one egg mass, remains poorly understood. Recent advances in digital imaging and computer-assisted morphometry provide new opportunities to transform qualitative traits into quantitative data. Image-analysis techniques have been successfully applied in entomology to measure shape, color, and pattern variation with high repeatability (Lehnert et al., 2011; Wen and Guyer, 2012; Belleghem et al., 2018; Høye et al., 2021). By digitizing morphological features, it becomes possible to apply robust statistical approaches, thereby improving objectivity and standardization in taxonomic and ecological research (Krell, 2004).

In this context, the present study aims to quantify variation in gula pigmentation among *Chironomus riparius* larvae derived from a single egg mass using image-processing techniques. By analyzing pigmentation area, intensity, and optical properties, we sought to (i) convert a traditionally qualitative character into measurable variables, (ii) assess the degree of intra-clutch variation under controlled laboratory conditions, and (iii) evaluate the potential of digital image analysis to enhance the taxonomic resolution and reproducibility of larval identification in *Chironomus*.

MATERIALS AND METHODS

Sample collection and rearing

An egg mass of *Chironomus riparius* was collected in May 2024 from a small pond located at 37°44'22.24"N, 29°05'47.70"E. The sample was transferred to the laboratory in a sterile Falcon tube filled with native pond water to prevent desiccation and mechanical damage. Identification of the egg mass followed the morphological criteria described by Branch (1931). Species confirmation was achieved through larval characters following Vallenduuk and Moller Pillot (1997) and verified by pupal exuviae identification based on Langton (1991); Wilson and Ruse (2005). A rearing procedure modified from Abashy (2005) was applied. The egg mass was placed in a 500-mL sealed rearing container equipped with an air compressor and maintained at ambient laboratory temperature. Water from the sampling site was filtered through a Whatman GF/A 47-mm filter using a Sartorius–Stedim polycarbonate filter holder to remove coarse organic matter. The rearing container was lined with strips of blotting paper to facilitate larval attachment and tube construction. Larvae were fed ad libitum with commercial tropical fish food throughout development. All cultures were maintained at a constant laboratory temperature of 23–24 °C. It was observed that the first larvae emerged from the egg masses about one week after oviposition, with complete separation occurring within the first two weeks; between the second and fourth weeks, the majority of larvae progressed to the fourth instar stage. The physico-chemical parameters of the pond water are summarized in Table 1.

Table 1. Physico-chemical properties of the water from which the egg mass was collected (SO: Saturation O_2, DO: Dissolved O_2, ORP: Oxidation reduction potential, WST: Water surface temperature, S: Salinity TDS: Total dissolved solids)

SO (%)	DO (mgL^{-1})	pH	ORP (mV)	WST (°C)	Salinity (‰)	TDS (mg L^{-1})
66.8	5.67	8.87	-119	24.0	0.0	94

Imaging head capsules

Fourth-instar larval head capsules were examined to quantify pigmentation variation. Only head capsules remaining attached to pupal exuviae were selected to ensure accurate instar identification (Ergović et al., 2024). Specimens were imaged under uniform dome-lighting conditions using an Olympus CX31 binocular microscope, following the methodology of Kerr et al. (2008). All images were captured at 400× magnification and standardized exposure settings to ensure comparability across samples. In ventral view, the head capsule of *C. riparius* exhibits several diagnostic structures including the mentum, mandibles, ventromental plates, and the gula—a sclerotized ventral region located posterior to the subgenal areas. Variation in gula pigmentation intensity and distribution among individuals was visually evident before digital quantification.

Image processing and measurement parameters

Image preprocessing was performed in GIMP 2.10 (GIMP, 2019) to remove the background and enhance contrast. Quantitative analyses were then conducted in ImageJ v1.53 (Schneider et al., 2012). The gula was delineated anatomically as the area between the mentum and post-occipital margin (Vallenduuk and Langton, 2010). Nine image-derived parameters were calculated (Table 2) to quantify both pigment quantity and optical properties.

Table 2. Parameters derived from image analysis

Parameter	Definition
Total Gula Area (TGA)	The area between mentum and post occipital margin (Vallenduuk and Langton 2010)
Pigmented Area (PA)	The area where the pigmentation boundaries are determined on the gula using threshold technique in image processing methods
Total Intensity (TI)	The intensity of total gula area
Pigment Intensity (PI)	The intensity of gula pigmented area
Pigment Ratio (PR)	The ratio of gula pigmented area to total gula area
Intensity Ratio (IR)	The ratio of gula pigmented area intensity to total gula intensity
Optical Density (OD)	The optical density of gula area
Pigment Optical Density (POD)	The optical density of gula pigmented area
Pigmentation Profile (PP)	The profile section of the pigmentation that extends from one edge to the other at the widest width of the gula

Image processing

All intensity-based measurements were obtained from 32-bit images, while optical-density analyses used 8-bit grayscale images. A Kodak No. 3 step-density tablet (scanned using an Epson Expression 1680 Professional scanner) was used for OD calibration (Figure 1), following the Rodbard four-parameter function in ImageJ. Calibration curves were generated prior to each measurement series to ensure accuracy. The actual OD coefficients measured for the density step tablet on the computer are given in the Table 3. OD calibration processes were performed in ImageJ software and the Rodbard calibration curve was generated by the software (Figure 2).

Figure 1. OD step tablet used for calibration (www.imagej.net)

Table 3. OD readings from white to black, and corresponding calibration values (Calibration process should be repeated in every application)

No.	1	2	3	4	5	6	7	8	9	10
OD	252.32	224.54	192.09	154.6	125.78	101.22	79.54	62	48.29	37.16
Value	0	0.06	0.2	0.34	0.49	0.64	0.79	0.94	1.1	1.26
No.	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	
OD	29.22	23.03	17.86	14	10.94	8.55	6.95	5.79	4.9	
Value	1.26	1.41	1.56	1.7	1.85	2.01	2.16	2.32	2.46	

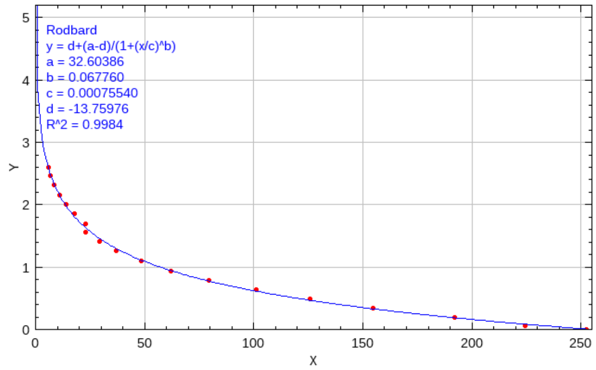

Figure 2. Calibration curve generated using the Rodbard function in ImageJ (The graph depicts the relationship between known concentration or size values (X-axis) and corresponding pixel intensity measurements (Y-axis). The curve is fitted with the four-parameter Rodbard equation. a: top asymptote, b: slope factor, c: inflection point, d: bottom asymptote)

Pigmentation profile analysis

Pigmentation profiles (PP) were extracted from each head capsule using the "Plot Profile" tool in ImageJ. Each transect comprised an average of 350 pixels across the widest part of the gula, producing a continuous intensity curve for each specimen. These data provided a spatial representation of pigmentation distribution along the ventral head capsule. An example of PP is shown in Figure 3. Based on an average of 350 pixels per sample across 40 samples, a robust dataset was established for statistical analysis. To evaluate differences in PPs among head capsules, a one-way analysis of variance (ANOVA) was performed.

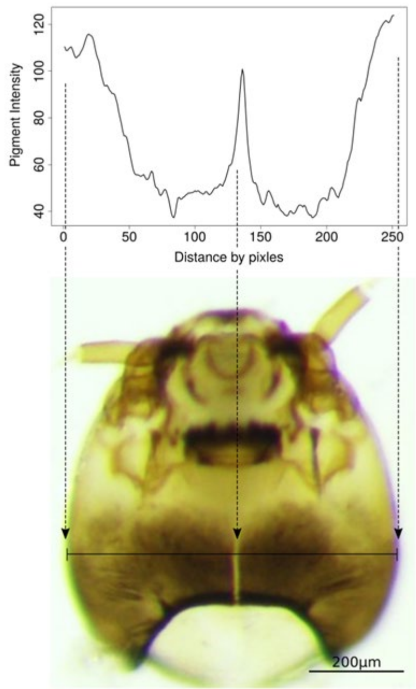

Figure 3. PP of the gula region in a larval head capsule (The profile illustrates variation in pigmentation density across the gula, which serves as a quantitative measure for comparative analysis. The lower image shows a ventral view of the head capsule, with a horizontal line indicating the transect along the widest part of the gula where pixel intensity was measured. The upper image displays the corresponding pigmentation intensity profile extracted using ImageJ software, representing pixel values along the transect)

Statistical analysis

All statistical analyses were conducted in R v4.1.0 (R Core Team, 2021). Descriptive statistics (mean, SD, range) were calculated for each variable. Normality was tested using the Shapiro–Wilk test, and data distributions were visualized with density plots and boxplots. Differences in PPs among individuals were tested by one-way analysis of variance (ANOVA). Pearson's correlation coefficients were calculated for normally distributed variables and Spearman's rank correlations for non-normal data. Correlation matrices and heat maps were used to visualize inter-relationships among pigmentation parameters, including area, intensity, and optical-density metrics.

RESULTS

A total of 40 fourth-instar *Chironomus riparius* larvae, all reared under identical laboratory conditions from a single egg mass, were examined to quantify variation in gula pigmentation (Figure 4). Visual inspection revealed distinct differences among individuals in both pigmentation intensity and the spatial extent of the pigmented region.

Descriptive statistics and normality

The summary statistics and normality test results for each variable are provided in Table 4. The total gula area (TGA) ranged from 72,247 to 112,364 µm² (mean ± SD: 90,442 ± 8,602.8 µm²) and conformed to normality (Shapiro–Wilk p = 0.849). In contrast, the pigmented area (PA) varied widely (16,803–70,857 µm²; mean ± SD: 36,688 ± 13,874 µm²) and showed significant deviation from normality (p = 0.007). Total intensity (TI) values ranged between 74.83 and 175.39 (mean ± SD: 139.39 ± 25.35) and exhibited a non-normal distribution (p = 0.025), while pigment intensity (PI) varied from 45.81 to 122.14 (mean ± SD: 86.74 ± 16.32) and followed a normal distribution (p = 0.734). The pigment ratio (PR = PA/TGA) ranged from 21.43% to 74.64% (mean ± SD: 40.30 ± 14.19) and significantly deviated from normality (p = 0.001). The intensity ratio (IR = PI/TI) spanned 44.00–86.99 (mean ± SD: 62.97 ± 9.70) and was normally distributed (p = 0.556). Optical density (OD) of the total gula area ranged between 0.055 and 0.341 (mean ± SD: 0.135 ± 0.057) and significantly departed from normality (p = 0.0002), whereas pigment optical density (POD) values (0.550–1.231; mean ± SD: 0.932 ± 0.174) did not differ from a normal distribution (p = 0.117). These results indicate that structural traits such as TGA, PI, IR, and POD exhibit relatively stable distributions, whereas PA, TI, PR, and OD display higher variability within the cohort (Figure 5).

Table 4. Minimum, maximum, mean, standard deviation and Shapiro-Wilk normality test results for measured parameters of gula (* p <0.05)

Parameter	Min.	Max.	Mean	Shapiro-Wilk Normality Test (p value)
TGA (µm2)	72247	112364	90442±8602.786	0.849
PA (µm2)	16803	70857	36688±13873.960	0.007*
TI	74.830	175.390	139.390±25.350	0.025*
PI	45.810	122.140	86.740±16.320	0.734
PR	21.430	74.640	40.300±14.190	0.001*
IR	44.000	86.990	62.970±9.702	0.556
OD	0.055	0.341	0.135±0.057	0.0002*
POD	0.550	1.231	0.932±0.174	0.117

Pigmentation profiles and intra-clutch variability

For each of the 40 larvae, an average of 350 pixel-intensity values was recorded along the widest transect of the gula to generate a PP. One-way ANOVA revealed significant differences among individuals in PP characteristics ($p<0.05$), confirming that pigmentation patterns varied significantly even within a single egg mass (Figure 4). No significant differences were detected in pigment intensity (PI) between individuals ($p>0.05$), and both PI and PI/TI ratio were normally distributed, indicating conserved pigment brightness across larvae. However, OD values measured across the entire gula region differed significantly among individuals ($p<0.05$), whereas POD values of the pigmented areas did not differ significantly ($p>0.05$). This pattern suggests that while the pigment substance itself retains consistent optical properties, the total light absorption of the gula varies due to differences in area and background tissue structure.

Correlation structure among pigmentation variables

Pearson's and Spearman's correlation analyses revealed distinct relationships among pigmentation-related parameters (Figure 5). A strong positive correlation was observed between PA and PR (r=0.97), indicating that larvae with larger pigmented regions also show proportionally greater pigmentation coverage. PA was moderately correlated with OD (r=0.65) and weakly with POD (r=0.24), implying that darker pigmentation accompanies increased area coverage. TGA waspositively correlated with PA (r=0.44) but negatively correlated with TI (r= −0.62), suggesting that larger gulas tend to exhibit reduced overall brightness. Total intensity (TI) showed strong negative correlations with both OD (r = −0.64) and POD (r=−0.55), reflecting the inverse relationship between reflectance and pigmentation darkness. PI was positively correlated with IR (r = 0.73) but negatively with OD (r=−0.38) and POD (r=−0.46), indicating that increased pigment intensity does not necessarily correspond to higher optical density.

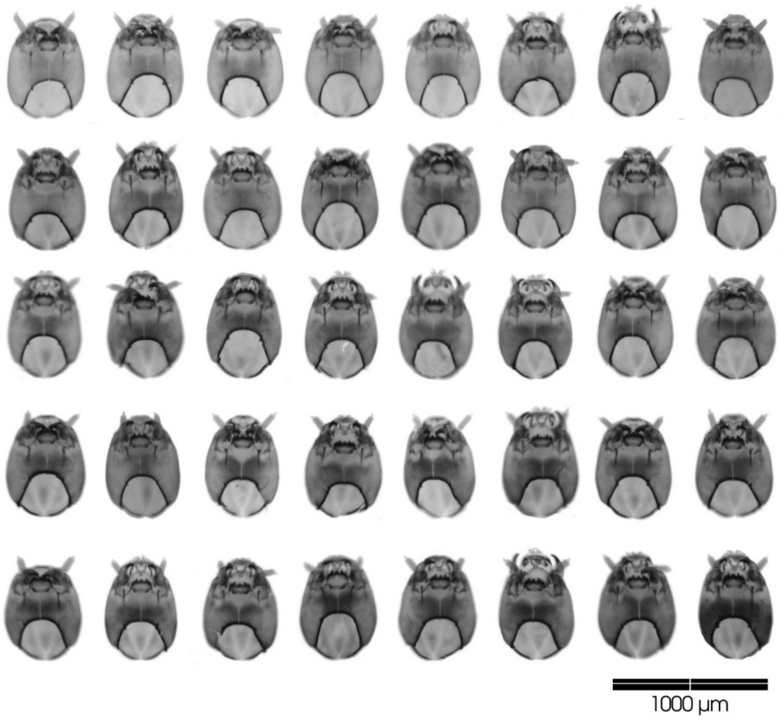

Figure 4. Ventral view of head capsules from 40 fourth-instar larvae of *Chironomus riparius* (All specimens were reared under identical laboratory conditions from a single egg mass and photographed under dome lighting at constant exposure and magnification (400×). Each head capsule was processed and scaled using ImageJ. The gula (central ventral sclerite) is clearly visible and exhibits variation in pigmentation intensity and area among individuals. Other identifiable structures include the mentum, mandibles, ventromental plates, and antennal bases. Post-occipital margins were excluded from the analysis)

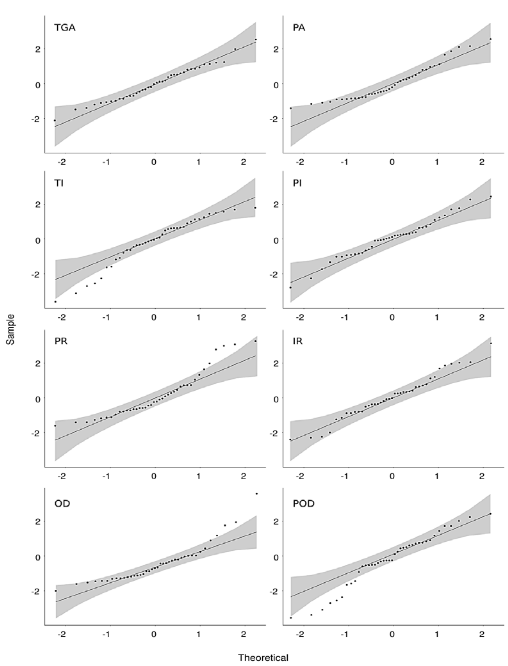

Figure 5. Visual inspection of the distribution of samples on the measured parameters (The Y-axis (samples) shows the quantiles of the samples, the X-axis (theoretical) shows the theoretical quantiles of the normal distribution (see Table 2 for short names of the parameters)

Overall, the correlation network highlights three principal axes of pigmentation variation: (1) Structural dimension (TGA, PA) — determining the extent of pigmentation, (2) Reflectance dimension (TI, PI, IR) — characterizing pigment brightness, (3) Absorptive dimension (OD, POD) — representing optical properties. These axes collectively illustrate that pigmentation variability in *C. riparius* larvae reflects a combination of morphological size, pigment concentration, and tissue optical differences. Despite being genetically identical and reared under identical conditions, larvae displayed significant intra-clutch variation in gula pigmentation metrics. Some parameters (e.g., PI, POD) appeared developmentally constrained, while others (e.g., PA, OD) exhibited stochastic or plastic variation. Tests for homogeneity of variance confirmed that variability across individuals was non-uniform, implying the presence of developmental noise or subtle environmental microgradients even under controlled laboratory conditions.

DISCUSSION

This study provides a detailed quantitative assessment of gula pigmentation variation in *Chironomus riparius* larvae using standardized image-processing techniques. By analyzing larvae derived from a single egg mass and reared under uniform laboratory conditions, genetic and environmental variability were effectively minimized. Consequently, the observed differences in pigmentation reflect primarily intra-clutch phenotypic variation rather than external influences. The observed variability in optical density may partly reflect microstructural differences in cuticular thickness and melanin deposition, as previously demonstrated in other dipterans and lepidopterans (Ninomiya et. al., 2006; Stavenga et al., 2014; Kownacki et al., 2018). The findings demonstrate that even within a genetically homogeneous cohort, pigmentation traits display complex patterns of stability and variability, offering insights into both developmental and taxonomic dimensions of chironomid morphology. Among the nine pigmentation parameters analyzed, structural variables such as total gula area (TGA), pigment intensity (PI), intensity ratio (IR), and pigment optical density (POD) followed normal distributions, indicating relative developmental stability. In contrast, parameters related to pigmentation extent and overall optical density (PA, TI, PR, OD) exhibited high variability and non-normal distributions. This divergence suggests that different biological mechanisms govern pigment synthesis, deposition, and distribution within the larval cuticle. The stability of PI and POD implies a conserved physiological control of pigment formation, whereas the variability in PA and OD may stem from fluctuations in cuticle thickness, microstructural arrangement, or differential chitinization rather than pigment concentration alone. The presence of statistically significant differences in the PPs among larvae from a single egg mass confirms measurable intra-clutch variability. Such variability likely reflects minor differences in developmental rate, metabolic state, or microenvironmental factors (e.g., oxygen or temperature gradients) experienced during larval growth (Frouz et al., 2002; Ward and Stanford, 2003). Moreover, the heterogeneity of variance among individuals indicates that pigmentation is not uniformly expressed across the cohort, a phenomenon consistent with stochastic developmental noise observed in other dipteran morphometric traits (Klingenberg, 2003; Wittkopp and Beldade, 2009).

The significant intra-clutch variability observed under controlled conditions may represent developmental instability, a phenomenon reflecting stochastic noise during morphogenesis (Palmer and Strobeck, 1986; Debat and David, 2001). Similar within-cohort asymmetries have been described in other insect taxa as indicators of developmental plasticity and canalization (Klingenberg, 2019). The observed combination of stable and variable pigmentation traits highlights a potential interplay between genetic regulation and developmental plasticity. Conserved parameters such as PI and POD may represent genetically canalized traits, reflecting fixed biosynthetic pathways for pigment production. Conversely, the plasticity of PA and OD could arise from environmentally sensitive or epigenetically modulated mechanisms influencing pigment deposition or optical properties of the cuticle. This interpretation aligns with theories of fluctuating asymmetry and stochastic gene expression that explain phenotypic heterogeneity in morphologically uniform populations. Pigmentation variability may also be influenced by subtle microenvironmental gradients such as oxygen or temperature (Pinder, 1986; Guillermo-Ferreira et al., 2014), suggesting a potential link between larval physiology and cuticle melanization intensity. In *C. riparius*, gula pigmentation may serve not only as a diagnostic feature but also as an indicator of physiological condition. Previous studies have associated pigmentation intensity and optical density with stress responses or contaminant exposure in aquatic invertebrates. The considerable range of OD and PA values even under controlled conditions underscores the need for baseline characterization of pigmentation variability before applying such traits as environmental biomarkers. Therefore, digital quantification provides an objective and repeatable framework for distinguishing between intrinsic variation and environmentally induced pigmentation changes.

Traditional morphological descriptions of *Chironomus* larvae often rely on qualitative evaluations of pigmentation, which are inherently subjective and prone to inter-observer bias. Quantitative image analysis, as employed in this study, aligns with recent methodological shifts emphasizing reproducibility and computational accuracy in morphometrics (Belleghem et al., 2018; Høye et al., 2021). The current approach, by contrast, transforms qualitative impressions into reproducible quantitative data through image-based measurement. This not only enhances the objectivity of taxonomic comparisons but also facilitates statistical analysis of character variation, potentially improving species discrimination among morphologically similar taxa. The correlation structure identified among pigmentation variables—linking area, intensity, and optical parameters—illustrates the multidimensional nature of pigmentation and may guide the selection of diagnostically stable metrics in future identification keys. The integration of digital morphometrics with conventional taxonomy represents a methodological advance with broad applicability. Beyond *C. riparius*, similar quantitative approaches could be employed to analyze coloration and structural features in other chironomid taxa or aquatic insect groups where pigmentation is taxonomically informative. Incorporating image analysis into larval identification protocols may thus contribute to higher reproducibility and transparency in ecological monitoring and systematics.

CONCLUSION

Gula pigmentation in *Chironomus riparius* larvae exhibits both conserved and variable traits, reflecting a balance between developmental constraint and plasticity. Quantitative image analysis has demonstrated its effectiveness in transforming traditionally qualitative features into robust, measurable parameters. This framework can serve as a foundation for future studies exploring the genetic, physiological, and environmental determinants of pigmentation in chironomids. Further research integrating gene-expression profiling, environmental manipulations, or cross-population comparisons would provide a more comprehensive understanding of the mechanisms underlying pigmentation diversity. Ultimately, the standardization of digital morphometric methods may enhance the diagnostic reliability of larval characters and advance both taxonomic resolution and ecological interpretation within the Chironomidae.

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AUTHORSHIP CONTRIBUTIONS

Gürçay Kıvanç Akyıldız: The author solely contributed to the conception and design of the study; data acquisition, analysis, and interpretation; development of methodology; visualization; drafting and revising the manuscript; and approved the final version for submission and publication.

CONFLICT OF INTEREST

The author declares that there are no conflicts of interest or competing interests.

ETHICS APPROVAL

No specific ethical approval was necessary for this study.

DECLARATION OF AI USE

No generative artificial intelligence tools were used at any stage of the preparation of this manuscript.

DATA AVAILABILITY

For questions regarding datasets, the corresponding author should be contacted.

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