Research Article

Association Between Egg Shape Index, Embryonic Sex, and Hatchability in KUB Chickens: A Morphometric Study with Molecular Validation

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Abstract | This study evaluated whether egg morphometric characteristics, particularly egg shape index (ESI), are associated with embryonic sex and hatchability in Kampung Unggul Balitbangtan (KUB) chickens. A total of 75 eggs were subjected to morphometric measurements using both manual methods and a MATLAB-based digital imaging system. Among these, 74 eggs were confirmed to be fertilised and 69 embryos were successfully sexed using CHD1 gene amplification as the molecular reference. Agreement between digital and manual measurements was assessed using Bland–Altman analysis, which demonstrated good agreement with a minimal systematic bias (mean difference = 0.003 ESI units). Mean ESI values did not differ significantly between male embryos (0.784 ± 0.027) and female embryos (0.792 ± 0.027) (Welch's t-test, $P = 0.279$; Cohen's $d = 0.27$). Receiver operating characteristic analysis yielded an AUC of 0.569 (95% CI: 0.423–0.708), indicating limited discriminative ability of ESI for predicting embryonic sex. No significant association was observed between ESI and hatching outcome (P = 0.653), and overall hatchability was 29.7%. This study did not detect a statistically significant association between egg shape index and embryonic sex in KUB chickens, although the relatively small sample size limits the precision of this estimate. Further studies with larger sample sizes and more controlled incubation conditions are required to clarify the potential role of egg morphometric traits in early sex prediction.

Keywords | egg shape index, In ovo sexing, Sex determination, Hatchability, CHD1 gene, KUB chicken

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INTRODUCTION

Local chicken are central to Indonesia's food security and smallholder farming economy. According to the Directorate General of Livestock and Animal Health (2024), the local chicken population reached 146.5 million in 2024, an increase of 47.88% compared to 2023. Egg and meat production from local chickens also increased significantly, reaching 236,560 tonnes of eggs (up 10.21%) and 156,450 tonnes of meat (up 8.81%) compared to the previous year. This growth underscores the economic importance of improving production efficiency in local

chicken systems, including through advances in early embryonic sex identification technology (Wibowo, 2016).

The Kampung Unggul Balitbangtan (KUB) chickens is an improved local breed developed by the Indonesian Agency of Agricultural Research and Development through systematic selective breeding. KUB chickens demonstrate improved productivity traits including reduced broodiness (10%), annual egg production of 130–160 eggs, age at first laying of 20–22 weeks, body weight of 1,200–1,600 g, and enhanced disease resistance (Sartika *et at*., 2013). These characteristics make KUB chickens valuable genetic resources for smallholder farming systems throughout Indonesia, particularly when raised under semi-intensive production systems rather than traditional free-range management. Compared with traditional unimproved local chickens, which typically produce fewer than 80 eggs per year under village production systems, KUB chickens exhibit substantially improved productivity, although their production levels remain lower than those of commercial layer strains raised under intensive management (Iskandar, 2005; Wibowo, 2016).

Early identification of chick sex could further improve production efficiency in such systems. The ability to determine sex before hatching can reduce the costs associated with rearing non-productive males, increase hatchery capacity for economically valuable females, and improve the efficiency of feed and labour allocation in small- to medium-scale poultry operations (Galli *et at*., 2017; Krautwald-Junghanns *et at*., 2018). In addition, in-ovo sex determination has gained increasing attention because it may help address ethical concerns associated with the routine culling of day-old male chicks in commercial layer production, a practice affecting billions of birds annually worldwide. However, the economic and ethical motivation for developing early sexing technologies does not by itself establish the biological feasibility of any indicator. Each proposed marker must therefore be independently evaluated for its predictive validity within the target population.

Among the non-invasive indicators proposed for early sex prediction, the egg shape index (ESI), defined as the ratio between egg width and egg length, has received considerable attention as a potential morphological predictor of embryonic sex. One hypothesis proposed in previous literature is that maternal hormone allocation during egg formation could influence egg morphology; however, empirical evidence supporting this mechanism remains limited. In birds, sex is genetically determined at fertilisation (ZZ in males and ZW in females), and sex-specific gene expression begins early during embryogenesis (Rutkowska and Badyaev, 2008). Some authors have hypothesised that sex-specific maternal hormone allocation, particularly variation in yolk steroid deposition, could influence oviduct physiology during egg formation and thereby affect egg geometry. Nevertheless, empirical evidence linking embryonic sex with egg morphology remains inconsistent across avian genus. While some studies report statistically detectable associations between egg shape and offspring sex, others find no relationship, suggesting that any effect may be weak, context-dependent, or dependent on breed-specific biological characteristics.

Kayadan and Uzun (2023) reported relatively high classification accuracy using ESI in specific poultry populations (hybrid chickens). However, morphometric associations identified in one breed cannot be assumed to generalise to other genetic backgrounds. Consequently, empirical validation is required for each population before morphometric indicators can be considered biologically meaningful or practically useful. Because KUB chickens originate from selectively improved indigenous populations rather than highly standardised commercial hybrid lines, differences in genetic background, production systems, and phenotypic variability may influence egg morphology and limit the direct transferability of morphometric findings across breeds.

Therefore, the present study aimed to evaluate whether egg shape index is associated with embryonic sex in Kampung Unggul Balitbangtan (KUB) chickens using molecular sex determination based on CHD1 gene amplification as the reference standard. In addition, this study assessed the agreement between manual morphometric measurements and a MATLAB-based digital image analysis system for estimating egg shape index. Rather than assuming predictive utility, this investigation treats the potential relationship between egg morphology and embryonic sex as an open empirical question requiring direct experimental evaluation.

MATERIALS AND METHODS

This study was conducted at the Reproduction Laboratory, Faculty of Animal Science, Brawijaya University, over a period of three months. All procedures performed in this study involving live animals were conducted in accordance with institutional ethical standards. The experimental protocol and animal handling were formally reviewed and approved by the Animal Ethics Committee of Faculty of Veterinary Medicine with Certificate No. 87-KEP-FKHUB-2026. Every effort was made to minimise animal suffering and reduce the number of animals used in the experiments.

Egg morphometric measurement

Hatching eggs were obtained from the KUB chickens

Hatchery (East Java Agricultural Modernisation Centre, Ministry of Agriculture). This location is 10 km from the laboratory. Eggs were collected from hens older than 35 weeks. All eggs were stored for 3 to 5 days before incubation in accordance with KUB hatchery procedures and transported in egg trays in a vehicle designed for transporting eggs. The total number of eggs was 75. Before incubation, the eggs were identified and given numerical IDs (1–75) and disinfected with 70% alcohol.

Each egg was weighed using a digital scale with a precision of 1 g. Morphometric measurements were taken using digital calipers with an accuracy of 0.01 mm. These linear measurements included the long axis (length (L)), short axis (width (W)), and longitudinal distance from the blunt end to the point of maximum width. The ESI was calculated as the ratio of egg width to length. A value close to 1.0 indicates a round morphology, while a lower value indicates a more elongated shape (Kayadan and Uzun, 2023).

Digital image processing

Morphological data for each egg were obtained through two integrated methods: direct physical measurement and digital image analysis (Figure 1). Physical dimensions, specifically the maximum width, were initially verified using a digital vernier caliper with an accuracy of 0.01 mm (Figure 1A).

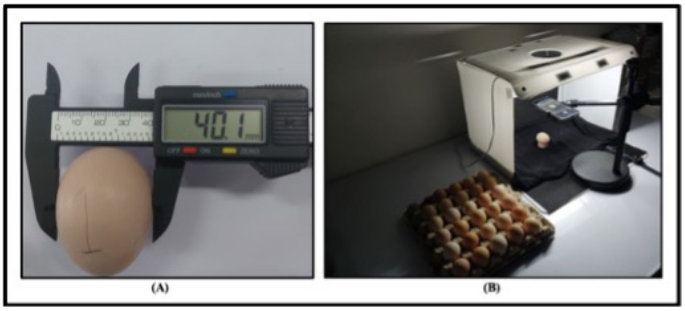


Figure 1: Egg morphometric measurement and digital image acquisition system used for estimating egg shape index (ESI). (A) Measurement of egg width using a digital caliper with a precision of 0.01 mm. The maximum width and egg length were used to calculate the egg shape index (ESI = width/length). (B) Digital image acquisition setup used for automated morphometric analysis. Eggs were placed individually on a stabilization ring inside a portable mini light-box studio equipped with integrated LED illumination and reflective interior surfaces to minimise ambient light interference. Images were captured using a smartphone camera mounted on a fixed stand at a constant distance of 15 cm to maintain consistent imaging geometry.

For digital acquisition, eggs were placed individually on a stabilisation ring at the centre of a portable mini-light box studio. This setup utilised integrated light-emitting diode (LED) lighting and reflective interior walls to eliminate ambient light interference and shadows against a high-contrast black background (Figure 1B). Images were captured using an iPhone 13 (Apple Inc., Cupertino, CA, USA) mounted on a stable adjustable stand at a fixed camera-to-object distance of 15 cm. To ensure image stability and prevent motion blur, the shutter was controlled wirelessly via an Apple Watch (Apple Inc., Cupertino, CA, USA).

All captured images were processed using MATLAB version R2021b (MathWorks Inc., Natick, MA, USA). The image processing workflow involved converting raw images to grayscale followed by binary segmentation to isolate the egg silhouette. Morphological features, including the long axis (length), short axis (width), and ESI, were extracted using the regionprops function (Zhang and Jacobs, 2025).

Chicken egg incubation

A total of 75 hatching eggs were incubated using a locally made still-air incubator (Figure 2) equipped with metal heating elements and a diffuser-type humidity controller. Environmental parameters were regulated by a digital controller (STC-3028) maintained at a temperature of 37.0 ± 0.5°C and a relative humidity of 60 ± 5%. The racks were arranged in a four-tiered system. After the ninth day of incubation, all eggs were examined to determine their fertility. The fertile eggs were placed proportionally as follows: top (n=19), upper middle (n=18), lower middle (n=19), and bottom (n=19). The eggs were turned twice a day until the 18th day of incubation. It should be noted that egg positions were not rotated across rack tiers during the incubation period; therefore, positional effects on thermal exposure cannot be ruled out as a contributing factor to hatchability outcomes. This represents a recognised limitation of the present study, as still-air incubators are known to exhibit temperature gradients of 2–5°C between rack positions (Barott, 1937; Moleenar *et al.*, 2011), and differential thermal exposure cannot be disentangled from intrinsic egg quality effects. Future studies should employ forced-air incubators with automated positional rotation to eliminate this confound. On day 19, the eggs were placed in individual cells to ensure strict tracking during hatching, enabling correlation of pre-incubation morphometric data with the specific sex of each chick determined in subsequent analyses.

Hatchability indicators were calculated from 75 eggs as follows: Fertilization rate = (No. of fertilised eggs / No. of laid eggs) × 100%; Hatch rate from fertilised eggs = (No. of healthy chicks/ No. of fertilised eggs) × 100%. It is important to note that ESI was measured prior to incubation, while sex was confirmed only after incubation ended (from hatched chicks or unhatched embryos). Egg shape may change during 21 days of incubation as a result of moisture loss and embryonic development (Wang *et al.*, 2019); therefore, any change in ESI between pre- and post-

incubation measurements was not tracked and represents an uncontrolled variable in hatchability analyses.

Sex determination methods

Genomic DNA was extracted from diverse biological samples including embryonic fluids from non-developing or dead embryos, feathers from hatched chicks, and tissues from unhatched eggs using a DNA Tissue Kit DC102-01 (Vazyme Biotech Co., Ltd., Nanjing, China) in accordance with the manufacturer's protocol. The use of multiple sample types was necessitated by the diversity of developmental outcomes; DNA extraction was successful in 69 of 74 fertile eggs, with 5 samples excluded due to extraction failure. Feather-based DNA extraction has been validated as a reliable approach for avian sex determination (England *et at*., 2021), while embryonic fluid and tissue samples were processed using the same validated kit protocol. Subsequently, PCR amplification was performed using primers SF (5'-GTGCATTGCAGAAGCAATATT-3') and SR (5'-GCCTCCTGTTTATTATAGAATTCAT-3') (Wang *et at*., 2019). These primers target the CHD1 gene and produce a single 500 bp band in males (ZZ) and two bands (350 bp and 500 bp) in females (ZW), exploiting the size difference between the Z- and W-linked CHD1 alleles. The CHD1-based method was selected because it is widely validated for avian sex determination and provides unambiguous genetic confirmation of embryonic sex (Iswati *et at*., 2021; England *et at*., 2021). No-template negative controls (NTCs) were included in PCR runs to verify reagent integrity. These controls consistently produced no detectable amplification bands under identical PCR condition, confirming the absence of reagent contamination throughout the experimental period. The reaction was prepared in a final volume of 15 µL, comprising 7.5 µL of Green Master Mix (GoTaq®; Promega Corporation, Madison, WI, USA), 0.5 µL of each primer, 1 µL of template DNA, and 5.5 µL of nuclease-free water.

Thermal cycling was conducted using a SensoQuest Labcycler (SensoQuest GmbH, Göttingen, Germany) with the following conditions: initial denaturation at 95°C for 3 min; 30 cycles of denaturation (95°C for 3 s), annealing (58°C for 30 s), and extension (72°C for 1 min); followed by a final extension at 72°C for 10 min. PCR products were resolved using a Microchip Electrophoresis System (MCE™-202 MultiNA; Shimadzu, Kyoto, Japan). Band visualisation was exported as electropherogram images and representative lane plots. Sex determination was based on fragment patterns, where a single peak indicated a male (ZZ) and two distinct peaks indicated a female (ZW). Any samples with ambiguous or faint band patterns were re-amplified independently before a final sex call was made.

Data analysis

All statistical analyses were performed using MATLAB R2021b (MathWorks, Inc.) equipped with the Statistics and Machine Learning Toolbox. Statistical significance was set at $\alpha = 0.05$. Agreement between manual caliper measurements and digital image-based measurements of Egg Shape Index (ESI) was evaluated using Pearson correlation analysis, paired t-tests, and Bland–Altman agreement analysis. Pearson correlation was used to assess the strength of linear association between the two measurement methods, while the paired t-test evaluated systematic differences between paired observations. Bland–Altman analysis was performed to assess agreement between the two measurement techniques by estimating the mean bias and limits of agreement. The observed proportion of male and female embryos was evaluated using a two-tailed binomial test to determine whether the sex ratio deviated significantly from the expected 1:1 distribution. Prior to hypothesis testing, the distribution of ESI values was assessed using the Shapiro–Wilk test together with visual inspection of quantile–quantile (Q–Q) plots. Homogeneity of variances between comparison groups was evaluated using Levene's test.

Differences in egg shape index between male and female embryos were analysed using Welch's t-test, which does not assume equal group variances and is appropriate for unequal sample sizes. The ability of egg shape index to discriminate between male and female embryos was evaluated using Receiver Operating Characteristic (ROC) analysis. The Area Under the Curve (AUC) and corresponding 95% confidence intervals were calculated using the MATLAB function perfcurve, with bootstrap resampling (1,000 iterations) to estimate confidence intervals. Differences in ESI between hatched and unhatched eggs were analysed using Welch's t-test.

RESULTS AND DISCUSSION

The results of morphometric validation, molecular sex determination, and statistical analyses are presented together with their biological interpretation to facilitate direct evaluation of the relationship between egg morphology, embryonic sex, and incubation outcomes in KUB chickens.

Validation of the digital image processing system

To evaluate the reliability of the digital imaging system, egg shape index (ESI) measurements obtained using MATLAB-based image analysis were compared with manual measurements obtained using digital calipers (Figure 1). Pearson correlation analysis revealed a very strong positive correlation between the two measurement

methods (r= 0.9708, P < 0.001) (Figure 4), indicating strong concordance in the relative ranking of eggs. Pearson correlation is widely used to evaluate the strength of linear association between two quantitative measurement approaches in morphometric studies (Mukaka, 2012).

A paired t-test detected a small but statistically significant difference between manual (0.789 ± 0.027) and digital (0.786 ± 0.029) ESI values (t = 2.64, df = 68, P = 0.010). The mean difference between methods was 0.003 ESI units, representing less than 0.4% of the mean ESI value, indicating that the magnitude of this difference is negligible in practical terms.

Agreement between the two measurement approaches was further evaluated using Bland–Altman analysis, which is widely regarded as the standard method for assessing agreement between two quantitative measurement techniques (Bland and Altman, 1986). This analysis revealed a small systematic bias, with digital measurements averaging 0.003 ESI units lower than manual measurements. The limits of agreement were narrow relative to the overall variability in ESI values, indicating that both methods produced highly comparable morphometric estimates across the observed measurement range.

To assess whether the observed offset could influence classification decisions, we evaluated its potential impact under hypothetical ESI threshold scenarios. Because the offset (0.003) was substantially smaller than the within-sample variability (SD ≈ 0.027), its influence on potential threshold-based classifications would be negligible.

The small systematic difference likely reflects minor calibration effects associated with pixel-to-millimetre conversion in the digital imaging workflow rather than substantive disagreement between measurement techniques. Because manual calipers measurements represent the established reference standard in previous morphometric studies, manual ESI values were retained for all subsequent analyses to ensure direct comparability with earlier work.

All manual measurements were performed by a single trained observer using standardised procedures to minimise measurement variability. Similar findings demonstrating strong agreement between digital image processing and manual egg morphometric measurements have been reported in poultry morphometry studies using automated image analysis systems (Kayadan and Uzun, 2023; Zhang and Jacobs, 2025). The validated digital system therefore provides an efficient tool for future high-throughput morphometric investigations.

Figure 2: Incubation system and egg arrangement during the experiment. (A) Interior view of the still-air incubator used in the study, equipped with analog thermometer and hygrometer to verify environmental conditions regulated by a digital controller. (B–E) Distribution of eggs across four incubation rack levels: (B) top tier, (C) upper-middle tier, (D) lower-middle tier, and (E) bottom tier. Eggs were distributed proportionally across tiers; however, positions were not rotated during incubation. Still-air incubators are known to exhibit temperature gradients between rack levels, which represents a limitation when interpreting hatchability outcomes.

Molecular sex determination and sex ratio

Molecular sexing using CHD1 gene amplification successfully identified the sex of 69 out of 74 fertile eggs (Figure 3), while five samples were excluded due to unsuccessful DNA extraction or PCR amplification. Among the successfully sexed embryos, 27 were identified as male (ZZ) and 42 as female (ZW), corresponding to a female proportion of 60.9%.

A two-tailed binomial test indicated that this deviation from the expected 1:1 sex ratio was not statistically significant (P = 0.091), suggesting that the observed skew most likely reflects sampling variability rather than a systematic biological bias within the population.

The use of CHD1-based molecular sexing provided a reliable reference standard for validating potential morphometric indicators of embryonic sex (Figure 5). Molecular sex determination targeting the CHD1 gene is widely used in avian studies because it enables accurate discrimination between Z and W chromosomes during early embryonic development (Griffiths *et al*., 1998; Fridolfsson and Ellegren, 1999).

Egg shape index and embryonic sex

Mean egg shape index (ESI) values were 0.784 ± 0.027 for male embryos and 0.792 ± 0.027 for female embryos (Table 1). Normality assumptions were assessed using Shapiro–Wilk tests and visual inspection of Q–Q plots, which indicated no substantial deviations from normality. Homogeneity of variance was confirmed using Levene's

test (P = 0.758). Welch's t-test revealed no statistically significant difference in ESI between male and female embryos (t = −1.094, df = 55.5, *P* = 0.279). The estimated effect size was small (Cohen's d = 0.27), suggesting only a minor difference in average egg shape between the two sex groups.

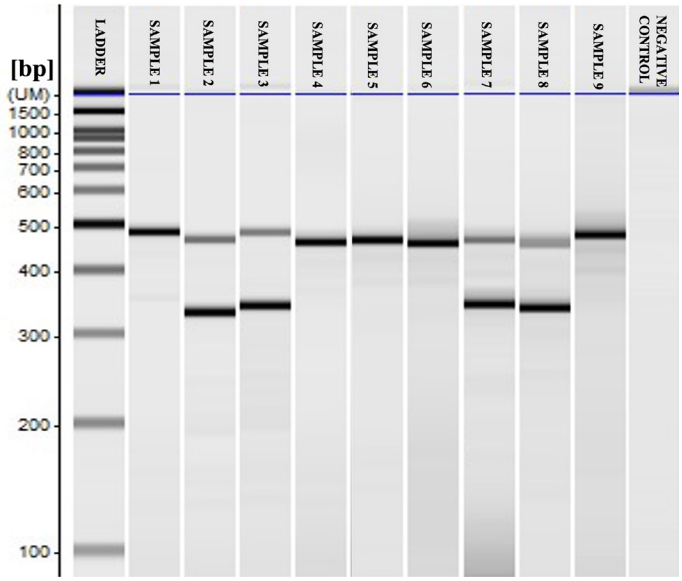
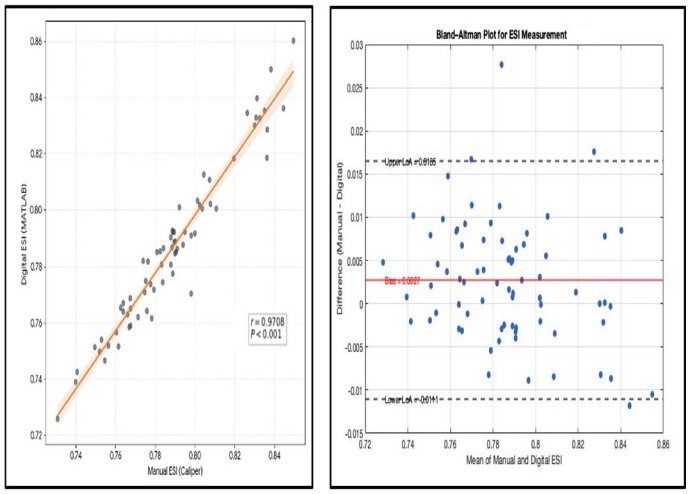


Figure 3: Molecular sex determination of chicken embryos using CHD1 gene amplification. Representative electrophoresis results obtained using a microchip electrophoresis system. The DNA ladder indicates fragment sizes in base pairs (bp). Male embryos (ZZ genotype) show a single band at ~500 bp, whereas female embryos (ZW genotype) show two bands (~350 bp and ~500 bp) corresponding to CHD1 alleles on the W and Z chromosomes. The negative control (NTC) shows no detectable amplification, confirming the absence of reagent contamination.

Receiver operating characteristic analysis (Figure 6) yielded an AUC of 0.569 (95% CI: 0.430–0.708). Although this value is slightly above the chance level of 0.50, the wide confidence interval indicates considerable uncertainty in the discriminative ability of ESI within this dataset. Therefore, the present study did not detect strong evidence that ESI alone can reliably discriminate embryonic sex in KUB chickens.

These findings are consistent with previous studies indicating that relationships between egg morphology and chick sex can vary considerably among poultry breeds and production systems. For example, morphometric classification models based on egg shape parameters have shown moderate predictive performance in some commercial poultry populations, although their general applicability across different genetic backgrounds remains limited (Kayadan and Uzun, 2023).

Egg morphology is determined by complex physiological processes occurring during egg formation, including oviduct contraction patterns, shell membrane deposition, and shell mineralization (Hincke *et al*., 2019). Because these processes are influenced by multiple biological and environmental factors, egg shape alone may not consistently reflect embryonic sex differences across breeds.



(A) (B)

Figure 4: Comparison between manual and digital egg shape index (ESI) measurements. (A) Scatter plot showing the relationship between ESI values obtained from manual caliper measurements and digital image analysis (n = 69). The solid line represents the regression fit with a 95% confidence interval. (B) Bland–Altman plot illustrating agreement between manual and digital ESI measurements. The central line represents the mean bias between methods, while the dashed lines represent the 95% limits of agreement.

Table 1: Comparison of egg shape index (ESI) between male and female KUB chicken embryos.

Variable	Male (n = 27)	Female (n = 42)	Statistical test	Statistic	P-value	Effect size
egg shape index (ESI)	0.784 ± 0.027	0.792 ± 0.027	Welch's t-test	t = −1.094 (df = 55.5)	0.279	Cohen's *d* = 0.27
			Mann–Whitney U test	U = 488.5	0.338	—
ROC analysis (female classification)	—	—	Area Under Curve (AUC)	0.569	—	95% CI: 0.430–0.708

Notes: Values are presented as mean ± standard deviation. Normality was assessed using the Shapiro–Wilk test and visual inspection of Q–Q plots, while homogeneity of variance was evaluated using Levene's test. Welch's t-test was applied due to unequal sample sizes between groups. The Mann–Whitney U test was performed as a non-parametric confirmation. Discriminative performance of ESI for sex classification was evaluated using Receiver Operating Characteristic (ROC) analysis with bootstrap confidence intervals (1,000 iterations).

Table 2: Comparison of egg shape index (ESI) between hatched and unhatched eggs.

Variable	Hatched eggs (n = 22)	Unhatched eggs (n = 47)	Statistical test	Statistic	P-value	Effect size
egg shape index (ESI)	0.791 ± 0.032	0.788 ± 0.025	Welch's t-test	t = 0.453 (df = 33.4)	0.653	Cohen's *d* = 0.12
			Mann–Whitney U test	U = 559.5	0.589	—

Notes: Values are expressed as mean ± standard deviation. Normality was evaluated using the Shapiro–Wilk test and Q–Q plot inspection, and homogeneity of variance was assessed using Levene's test. Welch's t-test was used due to unequal group sizes, and the Mann–Whitney U test was performed as a non-parametric verification.

Furthermore, the relatively small variation in ESI values observed in many chicken populations makes reliable sex classification based solely on egg shape statistically challenging. Consequently, the modest predictive performance observed in the present study is consistent with broader findings suggesting that egg morphology alone rarely provides sufficient discriminatory information for accurate sex prediction.

It is also important to consider that the present study had limited statistical power to detect small effect sizes due to the modest sample size. Therefore, while no statistically significant sex difference in ESI was detected, subtle morphological differences cannot be completely ruled out without larger datasets. Future studies incorporating substantially larger sample sizes will therefore be required to reliably detect small morphometric differences between sexes and to determine whether egg shape parameters contain biologically meaningful predictive signals.

Egg shape index and hatchability

An exploratory comparison of ESI between hatched and unhatched eggs was conducted; however, interpretation of this analysis should be cautious because incubation temperature gradients in the still-air incubator could not be fully controlled (Table 2). Hatched eggs exhibited a mean ESI of 0.791 ± 0.032, whereas unhatched eggs had a mean ESI of 0.788 ± 0.025. Welch's t-test indicated no statistically significant difference between these groups (t = 0.453, df = 33.4, P = 0.653; Cohen's d = 0.12).

Previous studies have reported inconsistent relationships between egg morphology and hatchability. A comprehensive review by Zhu et at. (2024) indicated that the association between egg shape characteristics and hatching success varies substantially across poultry breeds and production systems. Similarly, embryo viability during incubation is influenced by multiple physiological factors including albumen composition, shell quality, and antimicrobial protein activity that change throughout incubation independently of initial egg geometry (Biesek *et at*., 2023).

Breed-specific differences may further contribute to these inconsistencies. For example, studies on broiler breeder eggs have shown that hatchability may be optimised within a relatively narrow range of egg shape index values, suggesting that morphological–viability relationships are not universally transferable across different chicken populations (Peşmen, 2025).

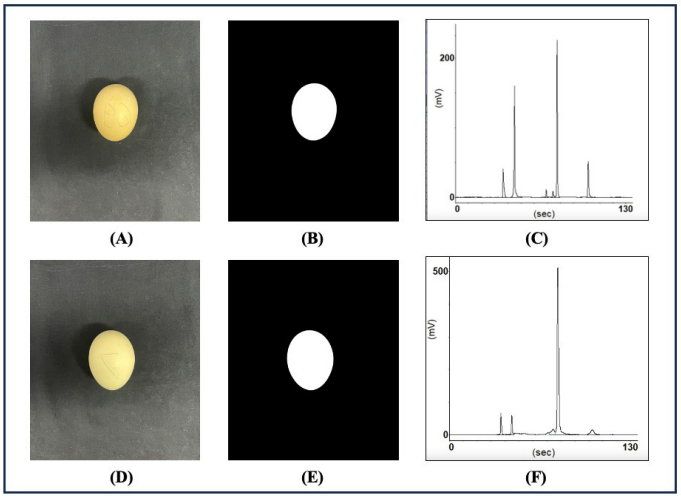


Figure 5: Representative examples of digital image segmentation and molecular confirmation of embryonic sex. (A, D) Original egg images acquired under standardised lighting conditions. (B, E) Segmentation output generated using the MATLAB-based image processing algorithm, illustrating the extracted egg silhouette used to estimate morphometric parameters including egg shape index. (C) Electropherogram showing two peaks (~350 bp and ~500 bp) corresponding to a female embryo (ZW genotype). (F) Electropherogram showing a single peak (~500 bp) corresponding to a male embryo (ZZ genotype).

In the present study, overall hatchability was relatively low (29.7%). Such reduced hatch success is unlikely to be explained solely by egg morphology and may instead reflect environmental factors associated with incubation conditions. Temperature variation within incubation systems has been shown to influence embryonic development and hatchability outcomes, particularly when eggshell temperatures deviate from the optimal range of approximately 37.8–38.2 °C (Lourens *et at*., 2005; Romanini *et at*., 2013). Consequently, the absence of an ESI–hatchability association should be interpreted cautiously.

Implications for morphometric sex prediction

Taken together, the present findings indicate that egg

shape index alone provides limited predictive information for embryonic sex determination in KUB chickens under the conditions of this study. However, this conclusion should not be interpreted as evidence that morphometric approaches are universally ineffective for poultry sex prediction.

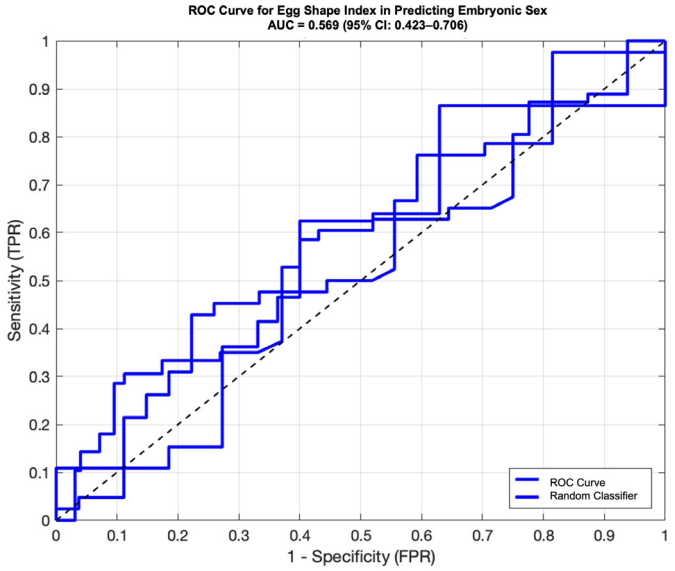


Figure 6: Receiver operating characteristic (ROC) curve evaluating the ability of egg shape index (ESI) to discriminate between male and female embryos. The diagonal dashed line represents the performance of a random classifier. The area under the curve (AUC) was 0.569 (95% confidence interval: 0.423–0.708).

The study provides several important contributions. First, it represents the first quantitative evaluation of the relationship between egg shape index and embryonic sex in KUB chickens. Second, it validates the use of a MATLAB-based digital morphometry system for measuring egg shape parameters, demonstrating strong agreement with manual measurements. Digital morphometric systems have increasingly been used for automated egg quality analysis and may facilitate large-scale phenotypic data collection in poultry breeding programs (Kayadan and Uzun, 2023; Zhang and Jacobs, 2025).

Recent research in poultry science has explored non-destructive analytical approaches for early embryo assessment, including optical imaging, spectroscopy, and machine-learning-based classification systems (Galli *et at*., 2017; Krautwald-Junghanns *et at*., 2018). Integrating digital morphometric features with these emerging technologies may improve the accuracy of early sex prediction models.

Future studies incorporating larger sample sizes, controlled incubation environments, and multi-parameter morphometric models will therefore be necessary to determine whether subtle morphological signals associated with embryonic sex exist in KUB chickens and whether such signals can be exploited for practical early sex prediction in poultry production systems.

CONCLUSION

This study evaluated the potential of egg shape index (ESI) as a non-invasive indicator of embryonic sex in Kampung Unggul Balitbangtan (KUB) chickens using CHD1-based molecular sexing as the reference standard. The results demonstrated strong agreement between digital image analysis and manual morphometric measurements, confirming the reliability of the MATLAB-based automated measurement system for egg shape assessment. However, no statistically significant association was detected between ESI and embryonic sex, and receiver operating characteristic analysis indicated limited discriminative performance of ESI within the present dataset. Similarly, no meaningful relationship between ESI and hatchability was observed. Because the sample size was modest and incubation conditions were not fully controlled, the present findings should be interpreted cautiously. Overall, the results suggest that ESI alone is unlikely to provide reliable early sex prediction in KUB chickens, although digital morphometric systems remain valuable tools for high-throughput egg characterisation and may support future studies integrating multiple egg traits or advanced analytical approaches for non-invasive sex prediction. Future studies integrating larger datasets, controlled incubation systems, and multi-parameter morphometric features may help clarify whether subtle morphological signals related to embryonic sex exist in KUB chickens.

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NOVELTY STATEMENT

This study provides the first empirical evaluation of the relationship between egg shape index (ESI) and embryonic sex in Kampung Unggul Balitbangtan (KUB) chickens using CHD1 gene-based molecular sexing as a reference standard. In addition, it validates a MATLAB-based digital image analysis system for egg morphometric measurement, demonstrating strong agreement with manual

methods. Unlike previous studies that assume predictive capability, this study rigorously tests the discriminative power of ESI using ROC analysis and shows that ESI alone has limited predictive accuracy for embryonic sex. These findings highlight the importance of integrating morphometric, molecular, and computational approaches for improving non-invasive in ovo sex determination in poultry.

AUTHOR CONTRIBUTION

MHY: Conceptualization, methodology, software, formal analysis, investigation, data curation, writing original draft, visualisation. FU: Methodology, software, validation. APAY: Investigation, resources. MHN, AAH: Supervision, project administration. TS: Conceptualization, supervision, writing review and editing, project administration, funding acquisition.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest

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