RESEARCH ARTICLE

Regular monitoring of the estrous cycle in rats for a sizeable experimental study

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ABSTRACT

Background: Hormonal fluctuations in female rats pose a challenge to their inclusion in experimental studies, resulting in their underrepresentation in biomedical research. However, monitoring the estrous cycle is essential for assessing reproductive cyclicity and detecting pathological changes. This study presents a standardized and optimized protocol for monitoring the regular estrous cycle in rats, addressing the labor-intensive nature and subjectivity of traditional vaginal cytology. *Materials and Methods*: The method involves systematic animal handling and sample collection, followed by crystal violet staining and microscopic examination to identify distinct cellular features of the four estrous phases: proestrus, estrus, metestrus, and diestrus. *Results*: Key improvements include pre-study acclimatization, standardized lavage collection volume, mixing samples for uniform smears, and preparing duplicate slides. These adjustments significantly reduced errors in estrus staging from 33.3 to 0.04% and cut procedure time by approximately 30%. *Conclusion*: The refined protocol improved data quality, reliability, and researcher confidence, proving effective for large experimental groups and minimizing animal stress. This comprehensive guide serves as a valuable resource for developing practical Standard Operating Procedures (SOPs) in laboratories conducting estrous cycle monitoring.

Keywords: Estrous cycle, Regular monitoring, Rat, Standard Operating Procedure.

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INTRODUCTION

Hormonal fluctuations in females of reproductive age are believed to be a significant limiting factor in the inclusion of female rats in experimental studies. A PubMed search for 'rats' returned more than 30,000 results for the last year, since June 2025, whereas the similar searches for 'male rats' and 'female rats' returned 20,000 and 5,000 results, respectively. This suggests an overall underrepresentation of female rats in biomedical research, as previously noted by some researchers [1-3]. On the other hand, the study of these hormonal fluctuations can serve as a marker of physiological reproductive cyclicity and of pathological or toxicological alterations [4].

The estrous cycle in rats, which typically lasts 4 to 5 days, is a repeated, dynamic physiological process characterized by complex hormonal changes, including the rhythmic release of estradiol, progesterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), as well as cellular alterations in the vaginal epithelium. This cycle is traditionally divided into four main phases: proestrus, estrus, metestrus, and diestrus. Each phase has a unique cell makeup visible in vaginal smears, which directly indicates the hormonal environment and the activity of the reproductive system (Table 1). Although the four-phase model is commonly accepted, some studies may use shorter or more detailed stages based on specific experimental requirements, particularly to accommodate the evaluation of transitional stages [4]. Female rats exhibit notable, stage-specific variations in various biological parameters, including gene expression, protein levels, and behavior, all of which are directly influenced by their current stage of the cycle [5]. The proestrus stage is in the follicular phase of development, characterized by rising estrogen levels, lasts 12 to 14 hours, and vaginal smears show mainly nucleated cells and erythrocytes. The estrus stage, during which rats exhibit sexual receptivity, is the ovulatory phase and lasts 9–15 hours; vaginal smears show primarily keratinized cells. Immediately followed by the metestrus stage, which lasts 21 to 24 hours, the corpus luteum begins to form in the ovary; during this stage, estrogen levels decrease while progesterone levels increase. Vaginal smears may contain keratinized cells, nucleated cells, and leukocytes. The diestrus stage has the most extended phase of the cycle, lasting 55–57 hours, during which the corpus luteum matures and secretes large amounts of progesterone to maintain the endometrium; vaginal smears show mainly leukocytes. This has been well standardized for over 50 years [5-7].

While traditional vaginal cytology remains a key method for detailed estrous cycle staging [5-7], its manual process is inherently laborious, time-consuming, and prone to subjective interpretation. Alternative approaches, such as vaginal impedance monitoring, provide a quick and less invasive option, although their primary use is in precisely identifying the proestrus stage. The development of

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automated estrous cycle staging, utilizing deep learning and advanced image analysis, marks a significant breakthrough, offering notable improvements in efficiency, objectivity, and accuracy for high-throughput research [5]. For large experimental groups, selecting and combining these methods requires careful consideration of practical factors, such as time, material and personnel costs, required expertise, and the implementation of robust standardization procedures.

When researchers need to monitor the estrous cycle regularly during a study, the main challenge is the extensive task of collecting, processing, staining samples, conducting microscopy, and maintaining records. Additionally, if a study involves four groups of female rats (a total of 24 rats) over 6-8 weeks, it is a demanding task for a single skilled researcher. For larger groups, the workload can be divided among 3 or 4 researchers; however, each can be assigned only one specific task throughout the study. High-level coordination, along with strict supervision, is necessary to ensure reliable data with 1000-1400 records. It is essential to follow ethical guidelines for animal welfare, prevent harm during sample collection, and remain unbiased during staging throughout the monitoring process.

To save time, reduce animal suffering, and improve data reliability, sharing tangible knowledge about standardizing the procedure is worthwhile. Therefore, the current study reports specific refinements in the estrous cycle monitoring process across a large group of animals.

MATERIALS AND METHODS

The reports presented here are derived from other studies where animals were procured for the evaluation of other specific hypotheses for which approvals from the Institutional Animal Ethics Committee were obtained. All procedures were conducted in accordance with the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA), Government of India.

Animal maintenance

For the current study, three different sets of 12 to 24 female, virgin Wistar rats (20–24 weeks old, 160–200 g body weight) were used across three attempts. The rats were bred and kept in a controlled environment (clean and noise-free, with a temperature of 23 ± 2°C and humidity of 50 ± 10%) in transparent polyvinyl chloride cages fitted with stainless steel grill covers. Three rats were housed in each cage, provided with commercially available chow and clean water (reverse-osmosed, with a total of 200 total dissolved ions) *ad libitum*. During the study, these eight cages were maintained alongside male rat cages on the same rack in a room with controlled airflow. The housing room was accessible only to the researchers involved, and the same team conducted all animal care and data collection.

Preparation

Once the animals are assigned for the study, the researcher begins comforting them with gentle handling, including holding the animals, exposing their ventral view, and providing easy access to the vaginal opening. This continued once daily for a week, which is equivalent to the initial acclimatization period when the animals are brought from outside the animal house setup. Feed consumption and water intake for each cage were recorded at the same time of day. By this time, the researcher should also have sufficient practice to hold the animal in an open-ventral posture with one hand and begin collecting the sample with the other. The comforting and sample collection procedures were conducted in a separate area within the room, so the process was not visible to the other animals.

Sample collection

The steps of sample collection are adopted from a published article [8] with some modifications. First, the genital is washed with distilled water, and the vaginal lavage from each rat is collected using a plastic Pasteur pipette. Sterile distilled water is taken up to one-third of the cylindrical tip of the Pasteur

Table 1: Key cellular characteristics of the rat estrous cycle phases

Features		*Phases of the estrous cycle in rats*			
		Diestrus	*Proestrus*	*Estrus*	*Metestrus*
Approximate Duration		48–72 hours	12–14 hours	24–48 hours	6–12 hours
Cytological characteristics	Predominant Cell Types	Predominantly leukocytes (neutrophils)	Small nucleated epithelial cells (round, non-cornified)	Large anucleated cornified (squamous epithelial) cells	Mixed: Leukocytes, cornified epithelial cells.
	Other Cell Types	Few cornified and few non-cornified epithelial cells	Occasional cornified cells; low numbers of large epithelial cells	Numerous bacteria (adhered or free)	Rare nucleated epithelial cells may appear.
	Neutrophils	Predominant	Rare to absent	Rare to absent	High
Relative Cell Density		Low to moderate	Low to moderate	Moderate to high	Moderate to high

Partially adopted from published literature [8]

pipette (Volume ≈ 50 µL; Temperature ≈ 37°C). Then the tip of the Pasteur pipette is placed in the vaginal opening (≈ 5 mm deep inside the vaginal opening), and then the available fluid is recollected after three gentle flushes. Then the content of the Pasteur pipette is fully ejected (collecting approximately 20 to 30 µL of the sample) into a marked petri dish with a glass cover. After placing the animal back in its cage, the collected lavage sample is thoroughly mixed with 10 to 15 complete and gentle ejection strokes of the Autopipette. Immediately after that, a 5 µL sample is placed on two pre-marked slides, labeled with a glass pencil for the date of sample collection and sequence of rat numbers, and allowed to dry under cover (on a flat surface) without disturbance for 24 hours (Figure 1).

Staining procedure

The dried slides were stained using a slightly modified method described elsewhere [8], by dipping the slides with smears into 1% crystal violet for 2 minutes. After that, the slides are washed twice by dipping them in distilled water for 1 minute each (Figure 2). Then, those were dried and seen under a microscope.

Microscopy

First, the stained smears are examined under a 4× objective to assess smear uniformity. Then, the stained smears are examined thoroughly under a 10× objective for cell identification and stage characterization. At least five fields from a single sample were studied under 10× for grading different characteristics, as listed in Table 2 and Figure 3.

Recording

The accurate interpretation of vaginal cytology samples depends entirely on the quality of sample preparation. The stages of the estrous cycle are determined by the absence, presence, and relative proportions of three main cell types: nucleated epithelial cells (which can be small or large), anucleated cornified (keratinized) cells, and leukocytes (neutrophils). A common method for staging involves first checking for neutrophils: if they are dominant or consistently present, the stage is either metestrus or diestrus; if they are rare or absent, the stage is either proestrus or estrus. The observations under the microscope were recorded in the format shown in Table 2.

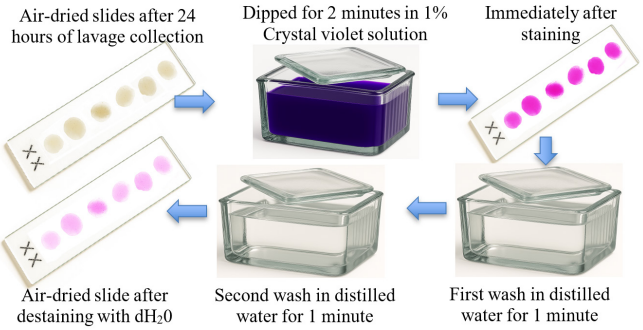

Figure 2: Step-by-step staining procedure. Images generated using Microsoft Copilot (Microsoft Corporation, 2025)

Each slide was coded (a diary was maintained for all samples) and evaluated by an observer who was blinded to the sample spot dates and sequence. The records from the two observers were compared weekly. When discrepancies arose, the sample spot was examined again under a microscope, and both researchers discussed their points to reach a consensus. Ultimately, the stage was confirmed based on the recorded estrus stages from the previous day and the following day for the same rat. If there was any uncertainty about the stage, a duplicate slide for the same sample (already preserved for all samples as mentioned in Sample collection) was used for confirmation.

RESULTS

Compared to earlier attempts to record the estrous cycle for 24 rats, this small but important adjustment, introducing specific refinements in the procedure details, helped us enhance data quality, researchers' confidence, and data reliability.

As shown in Table 3, during the first attempt at continuous estrus staging, the number of slides prepared exceeded the number of samples handled. This is because a substantial number of samples were to be duplicated, as the non-

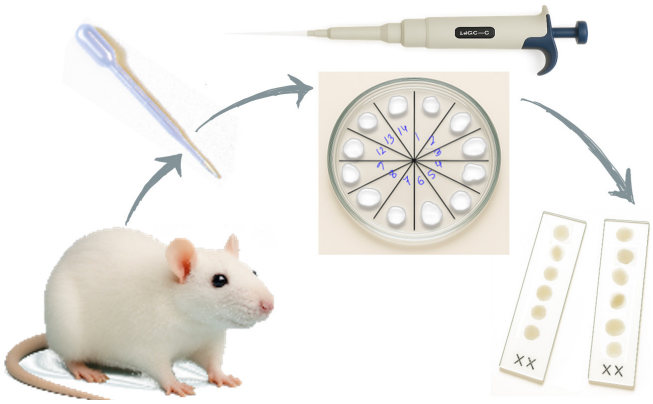

Figure 1: Collection of vaginal lavage and preparation of collective uniform smears in glass slides. Images generated using Microsoft Copilot (Microsoft Corporation, 2025)

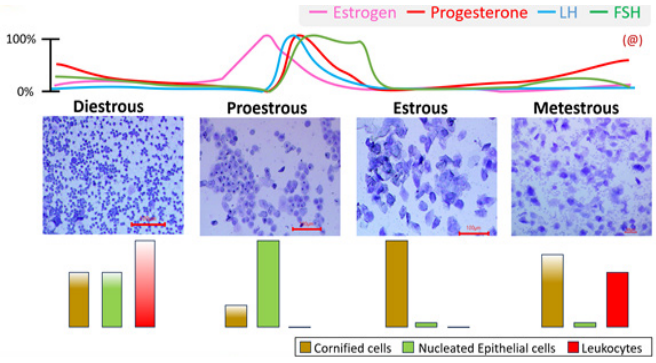

Figure 3: Sample microscopic observations with typical characteristics of different phases of the rat estrus cycle. @ Information used to draw the graphical representation of hormones is from Staley and Scharfman [9]

Table 2: Format for tabulation of reports obtained from microscopic examination of the stained smear and behavioral observations to determine the estrus stage of the rat.

A	B	C	D	E	F					G	H
Date	Mucus	Nucleated Epi. cells	Cornified cells	Other cells	Neutrophils percentage					Notable Behavior,	Estrus Stage –
	0/+/++	0/+/++	0/+/++	0/+/++	I	II	III	IV	V	if any	D/P/E/M
X1											
X2											
… …											

Column A: Rat number (X1, X2, etc.) and date of the sample. Columns B-E: Presence of mucus, nucleated epithelial cells, cornified cells, and other cells is noted as '0' = Absent, '+' = present a little or a few cells, and '++' = present in a significant amount. Column F: I to V indicates the presence of neutrophils with approximated grades of <10, 10-100, 100-500, 500-1000, and >1000 neutrophils per sq.µm of the field, respectively. Column G: Any change in behavior of the rat, like irritation, aggression, or depression, is noted on that specific day during the sample collection. Column H: The identified estrus stage (Diestrus, Proestrus, Estrus, and Metestrus).

Table 3: Comparison of attempts for estrous cycle staging in rats

Attempts	*Number of rats*	*Duration of monitoring*	*Slides [Samples] handled*	*Number of samples with problems (%)*			
				Mucus	*Inconclusive*	*Unwanted objects*	*Total*
One	12	14 days	207 [168]	16 (9.5)	34 (20.2)	6 (3.6)	56 (33.3)
Two	24	38 days	912 [912]	39 (4.3)	126 (13.8)	11 (1.2)	176 (19.3)
Three	24	52 days	231 [1248]	4 (0.003)	42 (0.03)	-	46 (0.04)

In all the attempts, the same researchers were involved in the staging procedure. Data presented in [] are the number of total samples processed, while data presented in () are the percentage values with respect to the total sample handled in that specific attempt.

confident handling of the rats led to animal irritation and, hence, improper vaginal lavage sampling, as evidenced by the physical nature of the collected lavage. This led to the same rat being sampled on the same day, further irritating the animals, and the sample became contaminated with urine and even blood.

Each collection takes approximately 10 to 15 seconds per rat once operators are proficient. The additional steps included in the current protocol may extend this time to 30 seconds per rat. The staining steps can take 1 to 2 hours or more for a batch of samples. However, the staining process described here can be completed within 5 to 10 minutes for dried sample specimens of a single day.

This step-by-step guide reduced the time required and the errors in determining a rat's estrus stage by nearly one-third. The briefing also boosts researchers' confidence in data collection. The noticeable improvements are not just due to adjustments and improvisations but also because researchers gain skills and experience through repetition. The noted mistakes and required improvements in the procedure are listed in Table 4.

During the Diestrus phase, leukocyte counts are variable. With experience, the number of leukocytes can be approximated, and based on that, the stage can be further subdivided into 5 phases, as proposed in Figure 4. The substages could be L1-L5, with roughly 5-10 times increments in observed

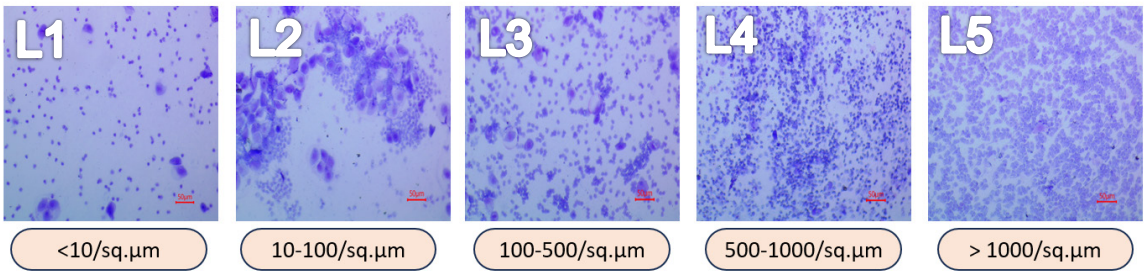

Figure 4: Sample snaps of microscopic observations for five levels of neutrophils during the diestrus phase of the rat estrous cycle

Table 4: Self-appraisal of the procedure followed by the researchers in the consecutive attempts to stage the estrous cycle in experimental rats

Mistakes / Improvements	*Comments*
Attempt one	
Did not start collecting the sample before the onset of the study.	No baseline data is available.
Animals were not comforted beforehand.	Handling stress for the animals was high.
Use of an Autopipette to collect the lavage	High risk of injury during sample collection.
More number of slides	More time spent on staining.
Attempt two	
Animals were handled more carefully.	Stress on the animals was noticeably less.
Sample collection began 10 days before the study's actual onset.	Baseline data is available before the study to confirm a normal estrous cycle.
Pasteur pipettes (plastic) were used to collect the samples.	However, the volume of the distilled water and depth of insertion were not standardized.
Samples were not mixed outside before the application on the slide.	All the collected lavage volume was applied to the slide, and eventually, more time was required to examine each slide.
Attempt three	
Comforting the animals and washing the genitalia before collection	Animals were less stressed, and the risk of contamination was also reduced.
Every day, before initiating sample collection, the Pasteur pipettes were marked with the volume of distilled water to be taken.	This marking helped achieve a relatively uniform lavage dilution volume. This marking also provided the researcher with an indication of the depth to which the tip reached.
Mixing the collected lavage before applying the specimen to the slides.	Uniform sampling of the collected lavage on both slides.
Each slide was marked with a pre-decided code and noted in the diary.	This coding helped to evaluate the observation by an unbiased researcher.
A small and fixed volume sampling.	Even distribution within the smaller specimen size. Less time was needed to study the specimen.
Each slide accommodated six lavage samples.	The number of slides was fewer, and the time required to stain them was shorter. Handling of specimens/slides was easier and regular.

leukocyte numbers. However, this is a preliminary suggestion based solely on the observations reported here, and proper validation is required for its further use.

DISCUSSION

As mentioned earlier, continuous monitoring of the estrous cycle is a crucial part of experimental studies to verify the normal reproductive functions of female rats, as well as in other studies where there is a risk of disrupting endocrine balance. Among the various methods, vaginal cytology is the 'Gold standard'. On the other hand, analyzing vaginal lavage is generally preferred over swabbing because it provides higher cellularity samples and minimizes cellular distortion, leading to better-quality smears.

The cyclical changes in vaginal cytology reflect rhythmic fluctuations in reproductive hormonal levels. Influenced by the ovarian sex hormones, female rats show distinct behavioral changes during specific phases of their cycle. Therefore, behavioral monitoring can be used as a supplementary indicator of estrus stages. However, due to its inherent subjectivity and the high likelihood of being influenced by environmental factors (e.g., the presence of males, stress), individual variability, and the specific experimental context, the support for behavioral expressions in estrus staging should be used with caution. On the other hand, the researcher should also be open to noticing changes in animal behavior to avoid unnecessary stress during sample collection and animal handling.

The method for collecting the lavage sample, analyzing it, and staging the estrous cycle is well standardized and followed by researchers worldwide. However, collecting samples and analyzing them regularly for a chronic experimental study

involving a sizable number of animals is a monumental task for a researcher. In this context, if a predefined protocol is established, the standardized details are likely to facilitate researchers in setting up and continuing the procedure smoothly. The current protocol for vaginal lavage-based estrus stage determination has improved the uniformity and reproducibility of the method by reducing human error at steps such as lavage collection, smear preparation, and evaluation of the smear to determine the estrus stage.

As noted, the errors in estrus staging decrease with each attempt. Apart from the standardization and corrections listed in Table 4, the experience gained and skills learned by the researcher during the process also played a crucial role in the reported refinement. Therefore, the most essential aspect of implementing the standardized protocol is the researcher's dedication and responsible involvement in the estrous cycle staging.

The debriefing and feedback from the research helped us reevaluate certain aspects of the process in terms of their importance. Providing a rationale for certain aspects of the current protocol may enable the researcher to decide whether to follow that part of the procedure and to perform the task more effectively.

Preparatory phase

This process should begin during the acclimatization phase, allowing at least two cycles to be completed to confirm the regularity, rhythmicity, and contiguity of the estrous cycles of each animal. After this, animals can be assigned to a study group.

Medium for lavage collection

For the current study, sterile double-distilled water was used as the medium for lavage collection; however, MiliQ water or sterile saline can be used, depending on availability and affordability.

Cleaning the genitalia

Washing the genitals with distilled water once confirms that the collected lavage is not contaminated with urine (uric acid crystals), as most rats feel stressed and urinate at the onset of any procedure (immediately after being held by the researchers), and may remain unnoticed.

Depth of tip to be inserted

It is better to avoid inserting the pipette tip too deeply (recommended depth of 5-10 mm for rats) to prevent cervical stimulation, which can inadvertently induce pseudopregnancy, manifesting as a persistent diestrus for up to 14 days.

Mixing the lavage before sampling

Absolute uniformity in the smear is not possible and cannot be confirmed. However, mixing the collected lavage sample in a petri dish before applying it to the slide can help rule out inadvertent sampling of the lavage portion devoid of its cytological features.

Duplicate slide preparation

It is recommended to prepare duplicate slides (as mentioned in the Method section) during the initial days. This duplicate slide can serve as a backup to replace spoiled samples during staining and analysis. Nevertheless, as the researcher becomes confident, the duplicate slide preparation can be omitted.

Multiple specimens on a single slide

This step provides uniform staining of all the specimens in addition to cost-cutting on slides and time-saving in staining and evaluation steps.

Fresh pipette and tip for each animal

To prevent cross-contamination and ensure data accuracy, a new Pasteur pipette and a fresh Autopipette tip must be used for each animal.

Debriefing and feedback

The researcher must remain involved and motivated throughout the entire process, from regular sample collection and processing to analysis and reporting. Regular debriefing and feedback between team members help them achieve this. Implementing estrous cycle monitoring in sizable experimental studies requires careful consideration of time, cost, expertise, and strategies to minimize variability and distress. The vaginal cytology method of estrus staging in rats is time-consuming. Sample collection must occur daily to assess cycle length and stage duration accurately. Evaluation requires careful examination of the entire smear under a microscope, which can be a lengthy process, especially for large cohorts. By introducing mixing and uniform small-volume sampling on the slide, the time required for microscopic evaluation was significantly reduced. The recurring cost of disposable sterile materials (pipettes, tips, slides, stains) for each animal over an extended collection period is significant.

Vaginal cytology requires substantial technical training and expertise to collect high-quality samples and accurately interpret cellular morphology. Identifying and differentiating among various cell types and recognizing artifacts (e.g., ruptured neutrophils) requires a trained eye. This qualitative interpretation protocol is a barrier for novice researchers and can lead to subjective judgments and inconsistencies, even among trained professionals. For sizable studies, development and strict adherence to detailed Standard Operating Procedures (SOPs) for all chosen monitoring methods, including sample collection, preparation, and interpretation criteria, are essential. Implementing rigorous training for new recruits is likely to ensure proficiency and minimize inter-observer variability. Regular quality control checks on interpretations should also be incorporated. The current protocol is believed to be a valuable starting point for developing SOPs for the laboratories involved in estrous cycle monitoring. This is likely to improve reproducibility across laboratories as well.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.