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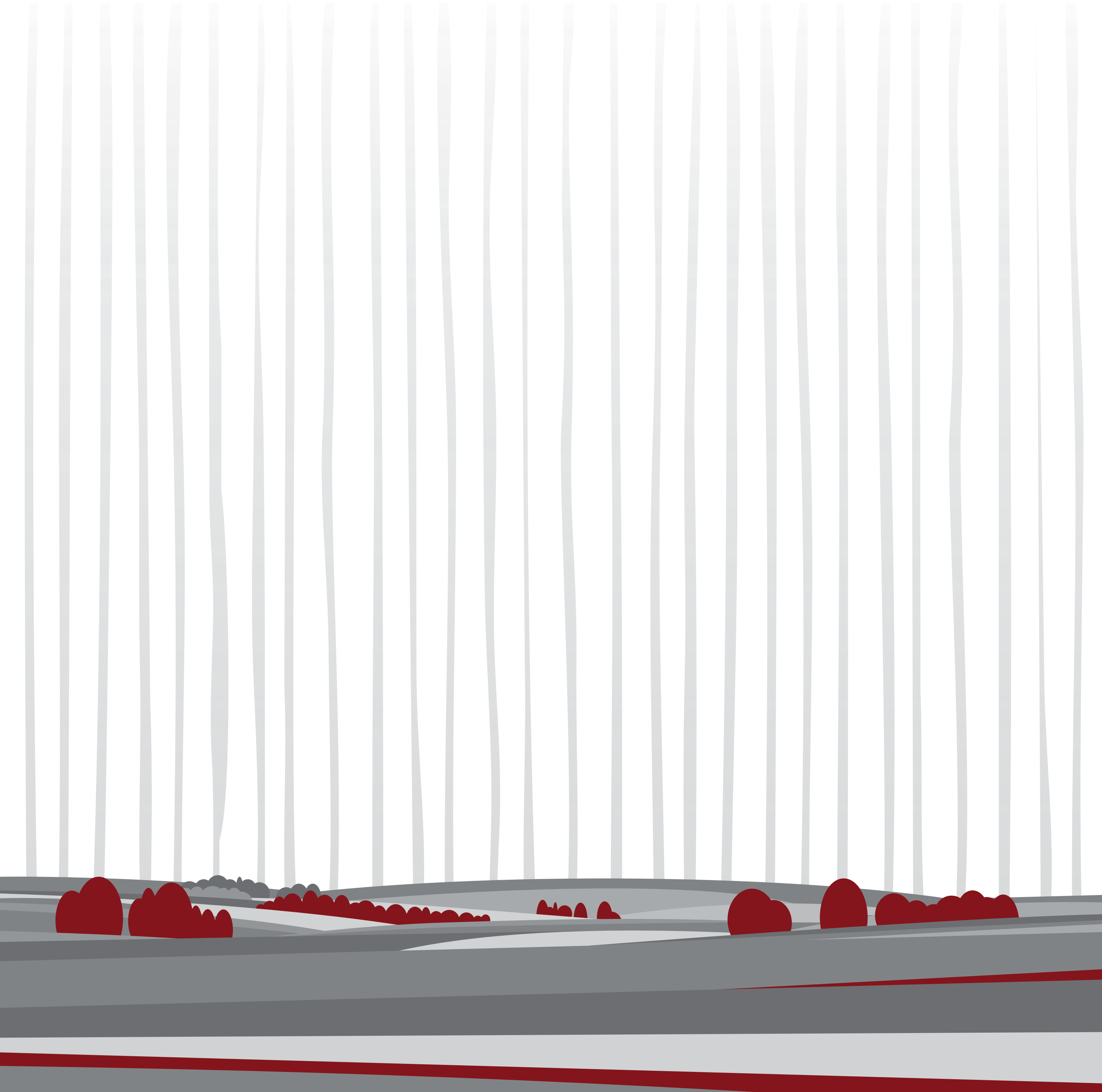

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An Autonomous Field Robot Farmbeast – The Field Robot Event 2023 Edition

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ABSTRACT

In contemporary agricultural automation, the demand for highly adaptive autonomous systems is rapidly increasing. Addressing this need, we introduce the latest iteration of FarmBeast, an advanced autonomous robot designed for precise navigation and operation within the complex terrain of cornfields. This paper details the technical specifications and functionalities of FarmBeast, developed by a Slovenian student team from the University of Maribor for the international Field Robot Event (FRE) 2023. The enhanced version features significant hardware and software upgrades, including a completely new robotic platform, a multichannel LIDAR system, an Xsens IMU, and advanced algorithms for efficient row navigation and weed removal. These integrated technologies aim to improve the efficiency and reliability of agricultural processes, reflecting the broader trend towards digitization and precision farming. Participation in international competitions like FRE provides a valuable platform for students to apply interdisciplinary knowledge, fostering the development of practical skills and understanding the interconnectedness of various scientific disciplines. As highlighted in the results section, FarmBeast performed notably compared to other 14 robots, securing top-five finishes in navigation, plant treatment, and obstacle detection tasks, demonstrating its capabilities in dynamic agricultural settings.

Keywords: precision agriculture, robotics, sensors, algorithms

INTRODUCTION

In the realm of contemporary agricultural automation, the demand for highly adaptive autonomous systems is rapidly increasing (Bogue, R., 2019). Addressing this need, we introduce the latest iteration of 'FarmBeast,' an advanced autonomous robot engineered for precise navigation and operation within the complex terrain of cornfields. This paper unveils the technical details and functionalities of FarmBeast, an autonomous field robot developed by the Slovenian student team from the University of Maribor for the international Field Robot Event (FRE) 2023. The enhanced version features significant hardware and software upgrades, including a multichannel LIDAR system, an improved power distribution PCB, and advanced algorithms for efficient row navigation and weed removal. The integration of these technologies aims to improve the efficiency and reliability of agricultural processes, reflecting the broader trend towards digitization and precision farming.

The development of autonomous agricultural robots, like FarmBeast, is part of a broader shift towards precision agriculture, which leverages digital technologies to optimize farming practices (Bose, P., 2020). Precision agriculture involves the use of various sensing systems, mobile applications, Internet of things (IoT), and other technologies to enable selective and precise treatment of crops. This approach leads to significant savings in input materials, reduces environmental impact, and increases crop yields (Rakun, 2023).

FarmBeast is an ongoing student project that started in 2018, based on initial Cornstar concept that started back in 2008 (Berk et al., 2016). The robot has undergone continuous improvements to enhance its capabilities in terms of speed, reliability, and robustness. In 2023, the robot base was completely rebuilt, providing a higher degree of stability and

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usefulness on the field. These enhancements are also supported by sophisticated software algorithms that utilize the LiDAR data for navigation and obstacle avoidance (Bernad et al., 2018).

Participation in international competitions like the Field Robot Event (FRE) provides a valuable platform for students to apply interdisciplinary knowledge from fields such as computer science, electrical engineering, mechanical engineering, and agricultural sciences. The integration of robotics in agricultural STEM education not only equips students with practical skills but also fosters an understanding of the interconnectedness of various scientific disciplines (Bernad, Rihter, & Rakun, 2024).

The objectives set by FRE 2023 challenge the participating robots to demonstrate their capabilities in five main tasks. These tasks are designed to test the robots' navigation, plant treatment, obstacle recognition, and overall performance in dynamic agricultural settings. The specific tasks include:

Navigation: Robots must autonomously navigate through a maize field, following a specified path while avoiding obstacles and maintaining accuracy and speed.

Treating (spraying) the plants: Robots must navigate and selectively treat plants with a spraying mechanism, demonstrating the ability to recognize gaps where plants are missing.

Sensing and recognizing possible obstacles: Robots are tested on their ability to recognize and classify obstacles such as humans and animals.

Static and dynamic obstacles: Robots must detect and appropriately respond to static and dynamic obstacles while navigating through the field.

Freestyle: Teams showcase their creativity and technical skills by presenting an innovative agricultural application of their robot, judged on originality, technical complexity, and performance (Field Robot Event, 2023).

Based on these objectives the paper is structured in the following way: section two describes the basic hardware and software of the FarmBeast autonomous robot, section 3 extends this; by describing the fundamental algorithmic principles, section four discusses the results and section 5 sums up the paper with selected conclusions and guidelines for future work.

MATERIALS AND METHODS

Robotic base

The FarmBeast robot, depicted in Fig. 1, is an advanced agricultural robot designed to automate the weeding and spraying process (Kajbič, M., 2023). This robot is equipped with a versatile attachment that allows for the targeted removal of weeds through three distinct methods: mechanical mulching, thermal elimination using lasers, and the application of various phytopharmaceutical preparations. Additionally, it includes a separate spraying device for in-row application, mounted on the back of the robot. The robot leverages machine vision and artificial intelligence to localize and identify weed species, optimizing the selection of the appropriate removal method. Designed for use within a single inter-row space, the FarmBeast also belongs to a broader category of agricultural robots capable of performing similar tasks across multiple rows simultaneously.

In terms of specifications, the FarmBeast robot features a modular design that allows for easy adjustments and the addition of various components as needed. It is equipped with a wheeled drive system that uses four-wheel Ackermann steering, providing high manoeuvrability, including the ability to turn around its geometric center. The robot is powered by DC brushless motors, which provide sufficient power to navigate slopes of up to a 50% incline. Its pneumatic suspension system can handle the robot's maximum load without exceeding 20% of the total suspension travel. The suspended part of the robot, along with the prescribed load capacity, totals 80 kg.

The design process for the FarmBeast robot considered several construction limitations. The robot must maintain a load capacity of 30 kg while ensuring that the suspended mass does not exceed 80 kg. The choice of a wheeled drive with Ackermann steering maximizes manoeuvrability, which is critical for operations in varied and often uneven agricultural terrains. The robot's modular construction allows for simple assembly and reconfiguration to adapt to different tasks, ensuring versatility in various agricultural applications. Additionally, the inclusion of an onboard compressed air source to power pneumatic actuators is essential for the operation of various agricultural attachments.

Extensive testing of the FarmBeast robot validated its design and functionality. All vital components met the required specifications during real-world tests, confirming the robot's robustness and reliability. The Ackermann steering system provided excellent manoeuvrability, allowing the robot to perform precise operations even in confined spaces. The pneumatic suspension system effectively maintained stability and traction, which is crucial for operations on uneven ground. Furthermore, the modular design and attachment mechanism proved effective, allowing for quick and secure changes of various agricultural tools.

The FarmBeast robot exemplifies a significant advancement in agricultural automation, combining machine vision, AI, and modular design to offer a flexible, efficient, and environmentally friendly solution for weed control. This robot's innovative features and robust design make it a valuable tool in modern precision farming, enhancing productivity while reducing environmental impact.

Figure 1: FarmBeast, an autonomous field robot

Sensors

The FarmBeast robot is a complex device equipped with multiple sensors, including encoders for wheel odometry, the Xsens Mti 610 IMU, the Velodyne VLP16 multichannel LiDAR, and the Realsense 435if RGBD camera. However, the core of the robot's navigation system relies on the synergy between two pivotal sensors: the Velodyne VLP16 LiDAR and the Xsens Mti 610 IMU. These sensors form the backbone of the robot's sensing and navigational intelligence, enabling it to respond with unparalleled accuracy to terrain challenges and the variable demands of precision agriculture competitions.

Velodyne VLP16 LiDAR sensor

The FarmBeast relies on the Velodyne VLP16 LiDAR sensor for its core spatial perception capabilities. Utilizing laser technology to create a semi-three-dimensional map of its surroundings, the sensor provides high-definition object and surface detection crucial for navigating through dense rows of corn. With the ability to capture 300,000 points per second, the VLP16 is essential for dynamic agricultural applications where reliability and precision are key to success.

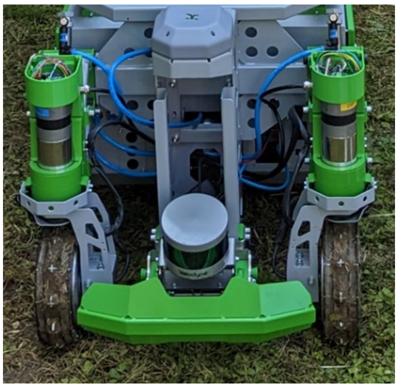

Figure 2: The front of the FarmBeast robot with a mounted Velodyne VLP-16 multichannel LiDAR

Xsens Mti 610 IMU

Complementing the LiDAR, the Xsens Mti 610 IMU delivers essential information about the FarmBeast's orientation and motion. Combining sensors for velocity, acceleration, and magnetic orientation, the IMU plays a crucial role in precise steering and stabilization during critical manoeuvres. The use of the Xsens Mti 610 is fundamental for executing the turns in patterns determined by competition organizers, ensuring that the robot maintains correct orientation relative to the complex geometry of the fields.

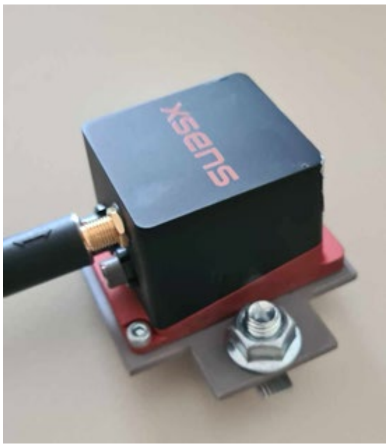

Figure 3: The Xsens IMU unit

Together, these sensors allow the FarmBeast to navigate autonomously and adapt swiftly to abrupt environmental changes, characteristic of competitive scenarios and real-world agricultural applications. This introduction lays the groundwork for an in-depth discussion on the innovations and technical solutions that FarmBeast brings to the field of agricultural robotics.

Field Robot Event 2023 - Tasks Overview and Scoring System

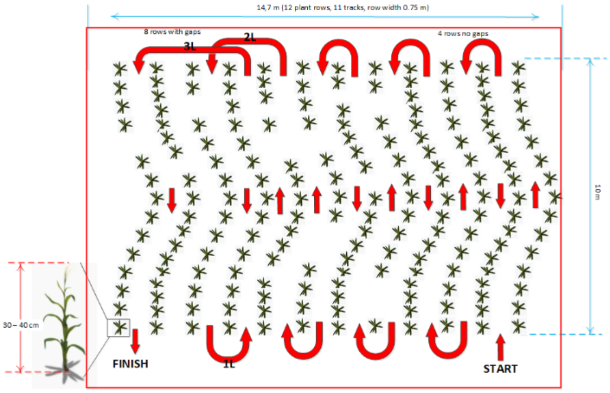

Figure 4: A map of the FRE field presenting the structure, plants and dimensions with the red arrows indicating the selected pattern for driving (source: FRE 2022 tasks description, www.fieldrobot.com)

This section describes in short, the focus of each task and presents the pointing system used in it. All 4 + 1 tasks had to be performed on the field, with specific goals set by each task. The Fig. 4 presents a basic information about the competition field, while Fig. 5 presents am image of actual conception and training fields from the FRE 2023.

Figure 5: An actual test field at the FRE 2023 held at the Faculty of Agriculture and Life Sciences, University of Maribor, Slovenia

Task 1: Navigation

Objective - Robots navigate autonomously through a maize field, following adjacent rows and a specific turning pattern after track 5.

Scoring - Distance travelled along the given path. A bonus factor for reaching the field's end in less than 3 minutes. Penalties for crop damage (2% of total row length distance per damaged plant).

Task 2: Treating (spraying) the plants

Objective - Robots navigate through the maize field, spraying plants when detected and stopping in areas without plants.

Scoring - Points for detecting empty regions and total distance travelled. Bonus for actual spraying accuracy, evaluated using water-sensitive paper (WSP) with weights based on WSP dryness. Penalties for crop damage and false positive detections (2% of total row length distance per damaged plant).

Task 3: Sensing and recognizing possible obstacles

Objective - Robots detect and classify obstacles (deer, human, unknown) from images placed in front of them. These images were submitted by all the competing teams, where only one image per team was then randomly selected. This resulted farness as the robots only detected on "familiar" image and the rest were classified by AI.

Scoring - 5 points for correct classification (true positive). -5 points for incorrect classification (false positive).

Task 4: Static and dynamic obstacles

Objective - Robots navigate the field while detecting and responding to static (deer) and dynamic (human) obstacles.

Scoring - Points for the path travelled (0.5 points per unit distance). 10 points for successful detection of obstacles. -10 points for unsuccessful detection of obstacles.

Task 5: Freestyle

Objective - Teams showcase their robot's capabilities in a creative performance related to agricultural applications.

Scoring - Points awarded for agronomic idea (originality), technical complexity, and robot performance (0-10 points each). The total points were calculated by:

$$Total_{points} = P1_{originality} + P2_{technical-complexity} \\ + P3_{\ performance}$$

Overall scoring

Points from each of the first four tasks are combined. Each task contributes up to 25% of the points for the overall assessment. Points for each task are calculated based on the ratio of points won by the team to points won by the winning team, adjusted to avoid negative scores.

ALGORITHMS

To enable the robot to achieve significant results, it must first navigate within a semi-predetermined field of corn. For this purpose, two main programs were developed: one for navigation through the field and another for turning the robot in specific patterns determined by competition judges a few minutes before the event.

The navigation program decides whether the robot's position needs correction by moving left or right within the corn maize. It begins by collecting data from the Velodyne VLP16 LiDAR sensor. These data points are filtered before use in the main navigation program through multiple layers of filters based on the RANSAC work of D. Kuramin (2023). First, noise is removed using a voxel grid, which down samples the original points by combining points within a specified area. Next, the normals are computed to perform ground plane segmentation. A RANSAC plane detection is also used to enhance ground detection with minimal jitter. The filtered point cloud data must be limited to the area we want the robot to detect. For this purpose, two areas in space were defined with a trapezoidal prism shape, fitting the dimensions of the competition rows. These areas are placed where we expect the middle of a corn row to be, and the remaining point cloud points in this area are assumed to be from the corn row, allowing us to compute the robot's required trajectory. The whole approach is summarized by a flow chart on Fig. 6.

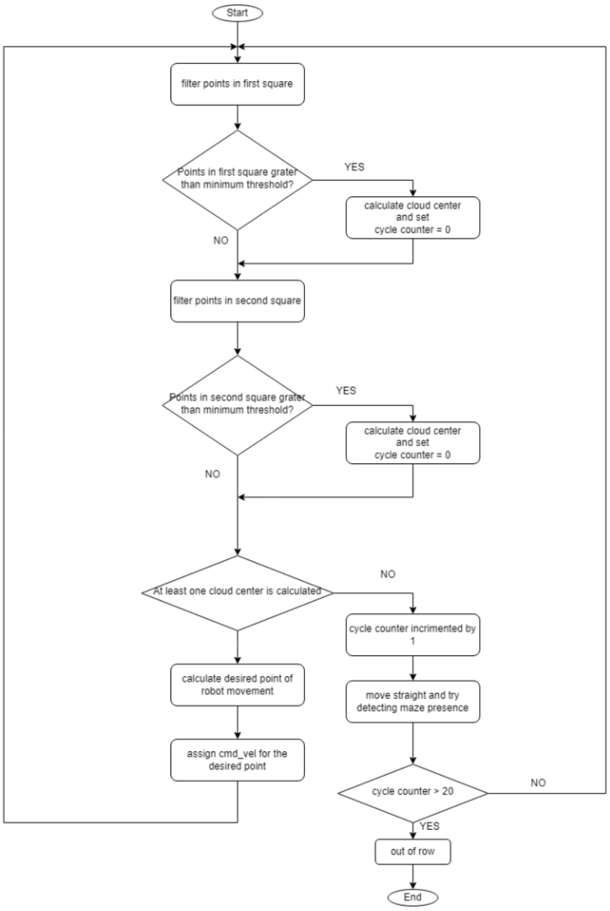

Figure 6: Flowchart of the navigational algorithm

First, the number of points remaining in each of the two areas is counted and compared to a minimum point threshold determined empirically. Based on these comparisons, different modes of operation are activated.

If both areas contain points, the average x and y positions of the points in each area are calculated, providing two center points to determine the robot's direction. The path correction logic is basic: an area is defined within which the robot can move straight, determined by the space available in the row for lateral movement. Given the robot's large size, this area was made small, roughly 4 cm.

If only one trapezoidal prism area has enough points, it indicates either a gap in the corn on one side or that the robot is at the maize's outer limit with only one side of corn. In this case, the center of the points in the populated area is mirrored to the other side to simulate both sides being full of points, allowing the robot to proceed accordingly.

If both areas lack points, it suggests the end of a row. However, the robot should not assume it is at the end of a row if both areas have points below the threshold, which can occur mid-row. To address this, a counter is implemented. If the robot detects no points in both areas for 40 consecutive cycles, it is assumed to be at the row's end. If points are detected in either area during any cycle, the counter resets. Upon reaching the end of the row, the robot switches to the turning program.

Turning of the robot

For turning, we use an Xsens IMU. The sensor provides data in quaternions (Hamilton, W. R., 1844), but due to time constraints and better comprehension, we initially implemented the program using Euler angles (Euler, L. 1776). Moving forward, we plan to switch to quaternions to make the code more concise.

The competition requires the robot to turn in a specific pattern, such as alternating left and right turns, but this pattern can change after a few iterations. The specific pattern is provided by the event organizers shortly before the task begins.

Our turning code is divided into two 90-degree segments to allow the robot to skip rows if needed. This involves an initial 90-degree turn, then the robot can skip one or two rows before completing the second 90-degree turn.

When the robot receives a turn command, it first gathers data from the IMU, converts it to Euler angles, and stores this orientation in a variable. Based on this value, we calculate the target orientation for a 90-degree turn to the left or right. For instance, if the current orientation is 130 degrees, a left turn would target 40 degrees, and a right turn would target 220 degrees. The robot checks the required direction and monitors for the corresponding orientation. To address occasional inaccuracies, we added a tolerance of ±5 degrees. When the robot is within this tolerance, the turn is considered complete. Any slight offset in orientation is corrected by the navigation algorithm. The calculations for offsets were straightforward for the second and third quadrants but more complex for the first and fourth quadrants due to the 0-360 degree transition, necessitating a more sophisticated algorithm.

State machine

An autonomous robot like FarmBeast runs multiple algorithms simultaneously, making it challenging to track which algorithms are active at any given time and potentially causing interference between programs. The solution to this issue is a state machine, which meticulously monitors state transitions and identifies the active programs. This approach also allows for a predefined sequence of algorithms, preventing unintended events and ensuring seamless coordination among various functions.

We used a ROS-based state machine called SMACH (ROS documentation, 2023), which offers graphical monitoring of the active state. This feature simplifies tracking active states and programs. The state machine updates ROS parameters, enabling interaction between different programs. At the end of each state, the current state variable is set to false, deactivating all algorithms used in that state, while the next state variable is set to true, activating subsequent algorithms. This setup significantly reduces the risk of the robot switching to an unwanted algorithm, such as initiating a turn in the middle of a row or activating the YOLO algorithm during a turning process.

Object detection and safety

Object detection is performed using the YOLO (You Only Look Once) algorithm (Redmon et al., 2016), a pioneering real-time object detection system highly regarded in computer vision. YOLO executes the entire object detection process in a single pass through the neural network, providing fast and accurate detection of objects in images or videos. By dividing the input image into a grid and predicting bounding boxes and class probabilities for each grid cell, YOLO minimizes computational redundancy, achieving remarkable efficiency without compromising accuracy. The basic principles of YOLO are illustrated in the Fig. 7 below.

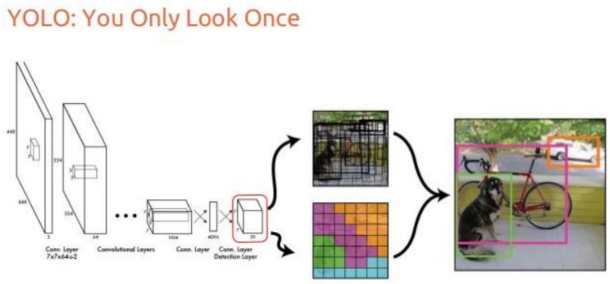

Figure 7: The basic principles of the YOLO algorithm (Analytics Vidhya, 2021)

YOLO's real-time performance has made it a cornerstone technology in applications such as surveillance, autonomous vehicles, and augmented reality. Each version of YOLO has introduced enhancements in speed, accuracy, and versatility, solidifying its status as a preferred solution for object detection tasks.

Effectively deploying the YOLO algorithm requires training neural networks on a comprehensive database of images to derive accurate weights. This process involves a substantial dataset of images containing the target objects, meticulously annotated to delineate the objects precisely. Additionally, a smaller set of test images is necessary to evaluate the accuracy of the trained weights. Although time-intensive, this preparatory phase is crucial for YOLO's efficacy. Once the weights are obtained through rigorous training, they can be integrated with the YOLO algorithm, enabling swift and accurate object detection in both images and video streams.

Initially, we searched online for available image databases to save time. We aimed to differentiate between people and deer, so we gathered a set of images for each and annotated the objects. We used approximately 800 images per category, totalling three detection categories: people, deer, and others. Images without deer or people were labelled as the third option.

Our algorithm employs the driving algorithm alongside wall detection, as the images we wanted to classify were positioned on a flat surface. We used the LiDAR sensor to detect this surface, pausing the driving algorithm momentarily for YOLO analysis. To ensure correct classification, we repeated the YOLO process five times. After classification, a speaker on the robot audibly announces the identified class. Depending on the class, the robot either continues driving if the object is removed or starts driving backward.

RESULTS

The FarmBeast robot participated in the Field Robot Event 2023, competing in five distinct tasks with 14 different international teams / robots. Each task was designed to test specific capabilities of autonomous agricultural robots, including navigation, plant treatment, obstacle recognition, and freestyle performance. The following sections detail FarmBeast's performance in each task, compared to other robots in real world operation, with points awarded for each placement. The videos of competing robots from the FRE2023 day one, two and three are added the reference section and present the competition in greater detail.

Task 1: Navigation

In Task 1, the robots were required to navigate autonomously through a maize field, following a predetermined pattern. The objective was to cover as much distance as possible within three minutes. The scoring system penalized any damage to crops.

FarmBeast achieved 4th place with a total of 80 points. Here are the results of the top five teams for Task 1:

Carbonite Schulerforschungszentrum Überlingen: 1st place (100 points)

- FREDT Technische Universität Braunschweig: 2nd place (90 points)
- Wageningen Robatic Bulls Eye: 3rd place (85 points)
- FarmBeast FKBV: 4th place (80 points)
- CERES Team and Team FloriBot Hochschule Heilbronn: 5th place (75 points)

Task 2: Treating (spraying) the plants

Task 2 required robots to autonomously navigate the maize field, spraying plants when detected and stopping the treatment in areas without plants. Points were awarded for accurate detection and treatment, with penalties for any missed detections or crop damage.

FarmBeast secured 5th place with a total of 75 points. The top five results were:

- Carbonite Schulerforschungszentrum Überlingen: 1st place (100 points)
- Wageningen Robatic Bulls Eye: 2nd place (90 points)
- FREDT Technische Universität Braunschweig: 3rd place (85 points)
- Karlsruhe KAMARO Betelgeuse: 4th place (80 points)
- FarmBeast FKBV: 5th place (75 points)

Task 3: Sensing and recognizing possible obstacles

In Task 3, robots had to detect and classify obstacles (a deer, a human, and other objects) from a set of images placed in front of them. Points were awarded for correct classifications, with penalties for misclassifications.

FarmBeast shared 4th place with Carbonite Schulerforschungszentrum Überlingen, both scoring 80 points. The competition was particularly tight in this task, with multiple teams performing well:

- Karlsruhe KAMARO Betelgeuse: 1st place (100 points)
- TH OWL: 2nd place (90 points)
- Milano Grasslammer, Osnabrück Team Acorn Acorn, TU Denmark DTU Maizerunners Thomas, and Wageningen Robatic Bulls Eye: 3rd place (85 points)
- Carbonite Schulerforschungszentrum Überlingen and FarmBeast FKBV: 4th place (80 points)

Task 4: Static and dynamic obstacles

Task 4 focused on safety, requiring robots to navigate the field while detecting and responding to static and dynamic obstacles. The difference beween the two was that the dynamic obstacles were removed, and the robot could continue, while the static did not and the robot had to move in reverse and continue in to the next row. Points were awarded for successful obstacle detection and avoidance, with penalties for failures.

FarmBeast achieved 5th place, scoring 75 points. The top five teams in this task were:

- TU Denmark DTU Maizerunners Thomas: 1st place (100 points)
- FREDT Technische Universität Braunschweig: 2nd place (90 points)
- Team FloriBot Hochschule Heilbronn: 3rd place (85 points)
- Wageningen Robatic Bulls Eye: 4th place (80 points)
- FarmBeast FKBV: 5th place (75 points)

Task 5: Freestyle

In the freestyle task, teams were invited to showcase their robot's capabilities in a creative and application-oriented performance. Points were awarded based on agronomic idea, technical complexity, and robot performance.

FarmBeast did not participate in the freestyle task due to technical issues. The top performers were:

- Wageningen Robatic Bulls Eye: 1st place (100 points)
- Karlsruhe KAMARO Betelgeuse: 2nd place (90 points)
- Osnabrück Team Acorn Acorn: 3rd place (85 points)
- FREDT Technische Universität Braunschweig: 4th place (80 points)
- CERES Team: 5th place (75 points)

Overall results

Combining the scores from all tasks, FarmBeast secured 5th place overall in the competition with a total of 310 points. The overall rankings are as follows:

- Carbonite Schulerforschungszentrum Überlingen: 1st place (450 points)
- Wageningen Robatic Bulls Eye: 2nd place (440 points)
- FREDT Technische Universität Braunschweig: 3rd place (435 points)
- TU Denmark DTU Maizerunners Thomas: 4th place (435 points)
- FarmBeast FKBV: 5th place (310 points)

CONCLUSION

FarmBeast's performance across various tasks highlighted its strengths in navigation, plant treatment, and obstacle detection. However, there is room for improvement in future competitions, particularly in the freestyle category.

The rapid advancements in agricultural robotics, as demonstrated by FarmBeast, underscore the pressing need for continued innovation in precision farming technologies. These technologies not only enhance productivity and efficiency but also reduce the environmental impact of agricultural practices. As the field evolves, it is crucial to develop new studies and job profiles that cater to the unique demands of precision agriculture.

Moreover, an educational system that integrates robotics, computer science, engineering, and agricultural sciences is essential to equip the next generation of professionals with the skills needed to drive this transformation. Interdisciplinary programs and hands-on training through participation in competitions like the Field Robot Event can provide students with invaluable experience, preparing them for future roles in this dynamic sector.

By fostering collaboration between academia, industry, and agricultural practitioners, we can ensure that the development of precision farming technologies continues to meet the challenges of modern agriculture. This holistic approach will not only advance the field of agricultural robotics but also contribute to sustainable farming practices that can meet the global food demands of the future.

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Avtonomni kmetijski robot FarmBeast: različica FRE2023

IZVLEČEK

Zahteve sodobnega kmetijstva narekujejo potrebo po visoko prilagodljivih avtonomnih robotskih sistemih. Kot enega izmed možnih odgovorov predstavljamo najnovejšo različico naprednega avtonomnega robota FarmBeast, ki omogoča avtonomno navigacijo in natančno delovanje v zahtevnih naravnih okoljih, kot so na primer koruzna polja. Članek opisuje tehnične specifikacije in funkcionalnosti robota FarmBeast, ki ga je razvila ekipa slovenskih študentov z Univerze v Mariboru z namenom sodelovanja na mednarodnem dogodku Field Robot Event (FRE) 2023. Izboljšana različica robota vključuje pomembne nadgradnje strojne in programske opreme, kot so povsem nova robotska platforma, uporaba večkanalnega LiDAR sistema, Xsens notranje merilne enote (IMU) in napredni algoritmi, ki omogočajo učinkovito navigacijo med vrstami ter funkcionalnosti za odstranjevanje plevela. Te integrirane tehnologije izboljšujejo učinkovitost in zanesljivost kmetijskih procesov, kar odraža širši trend digitalizacije in vse večjo uporabo tehnologij preciznega kmetijstva. Sodelovanje na mednarodnih tekmovanjih, kot je FRE, nudi pomembno platformo za študente, ki ob uporabi interdisciplinarnih znanj razvijajo praktične veščine ter razumejo medsebojno povezanost različnih znanstvenih disciplin. V članku so predstavljeni tudi rezultati evalvacije razvitih rešitev, kjer se je FarmBeast med 14 različnimi robotskimi sistemi odlično izkazal in se uvrstil med prvih pet ekip v disciplinah navigacije, obdelave rastlin in zaznavanja ovir, kar potrjuje njegove sposobnosti za uporabo v dinamičnih kmetijskih okoljih.

Ključne besede: precizno kmetijstvo, robotika, senzorji, algoritmi

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Evaluation of Heifer Welfare in Two Different Rearing Systems

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ABSTRACT

The welfare of domestic animals, which is a core part of livestock farming today, depends strongly on the rearing system. While free-stall systems are encouraged, tied rearing systems are still the most widely used in cattle farming, although considered less suitable due to restricted freedom of movement. As an alternative to this system, free-stall rearing, which allows movement, is proposed as a minimum standard. Although it seems self-evident that free-stall rearing is better than tied rearing system, there is still a lack of research on whether this is true for all categories of cattle or whether it might be appropriate for some of them (e.g. depending on age). The aim of the present study was therefore to compare the welfare of heifers in tied and free-stall rearing during the fattening period (at different ages). In general, we have suggested higher welfare scores in free-stall system, with the tied stall being more problematic for younger animals. The study comprised five heifers in each system per repetition (20 animals in total). Data were collected using the Welfare Quality® protocol, which involves the assessment of four main principles: feeding, housing, health and behaviour. The assessments were carried out approximately every three months during the fattening period (from 6 to 27 months of age). The results showed significant differences in animal welfare scores between tied and free-stall systems only in terms of housing and the tendency of differences in behaviour. For both principles, the scores were relatively low compared to feeding and health, where no differences were found between the rearing systems. In terms of temporal dynamics, differences were only found for housing, with values decreasing with age in both rearing systems. In the free-stall system, the scores were almost optimal in young animals (>90), but decreased rapidly with increasing age, while in the tied housing system, a suboptimal welfare scores were already observed in young animals (≈40). This means that tied rearing system is a clear disadvantage for the welfare of younger animals, which are generally more exploratory and active. Our results confirmed tied stall as less suitable in terms of animal welfare, especially for young animals. It is therefore encouraged that young animals should primarily be housed in a free-stall pens if both systems are available in the breeding facilities.

Keywords: animal welfare, cattle, heifer, rearing system, assessment protocol

INTRODUCTION

Animal welfare has become increasingly important in recent years as one of the elements of the overall concept of "food quality". Consumers expect food to be produced in compliance with animal welfare guidelines; as a result, animal welfare assessment protocols have been developed (e.g. Welfare Quality®, 2009). One of the fundamental aspects of animal welfare is whether animals have the opportunity to move freely. In this context, tied rearing system is considered problematic, unsuitable method of husbandry, as it restricts the animals' movement and makes it impossible for them to express their natural behaviours.

In 2020, the proportion of cattle in tied rearing in Slovenia was 73 %, i.e. in around 21,000 farms with almost 350,000 cattle and in around 4,300 farms (15 %) the free stall system (slurry-system) was used for fattening cattle (SURS, 2024). A definitive ban on tethering cattle is currently being discussed in the European Union. So far, only Norway and Denmark have decided to restrict this system. In Denmark, the ban will come into force on 01.07.2027, while Norway will introduce a ban on 01.01.2034 (Vešnik, 2023). In 2024, Austria also decided and agreed to ban this system after 2030. The date for the ban on tethering is repeatedly postponed, as tied rearing continues to dominate. A ban on tethering would displace many family farms. In 2007, for example, the EU decided to ban tied rearing on organic farms, which only came into force in 2014, but provided for an exception for small farmers. Therefore, agreements between breeders and politicians are being sought with new standards for this system, e.g. 245 days tethering and 120 days free movement (Expertise for Animals, 2024). Slovenia has not yet decided to

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restrict tied rearing, and so tied rearing will continue after 2027.

In tied rearing system, problems arise mainly due to the limited space, such as standing up and lying down. Space is limited by short stands and high mangers (Hoffmann and Rist, 1975). From this it can be deduced that the animals could be injured by inadequate housing. Urinating and defecating in the same place leads, among other things, to a higher proportion of dirt on the body parts (which is one of the measures of animal welfare quality), especially if the recommendations for daily cleaning of the barn are not followed (Whay et al., 2003). In addition, there are many other problems associated with tied rearing, such as an insufficient number of drinkers in the barn, which can result in subordinate cattle drinking less frequently than dominant cattle (Little et al., 1980). Leg problems and pressure sores are more common in older animals (due to high weight and lack of exercise) in a tied rearing system, while in a free-stall system, injuries can occur due to slippery floors and interactions between animals (more common in younger animals). Nevertheless, tied rearing is still widely used in animal husbandry, especially in cattle breeding. This system is attractive due to the low investment costs. It offers excellent individual care for the animals. Since the animals are at rest, it is also suitable for carrying out treatments, veterinary work and breeding (Morabito and Bewley, 2020). However, free-stall housing is generally considered as the better alternative for improving animal welfare, as it offers the animals more comfort as they can move around freely. Indeed, free-stall housing also has certain shortcomings. One of these is the higher risk of injury (Whay et al., 2003), which can result from negative social interactions (aggression, etc.) that animals may engage in when kept in free-stall pens and which can be harmful when space for movement is limited. In this respect, access to pasture can have a positive effect on cattle health and behaviour (Von Wachenfeldt, 1997). It is difficult to always plan for the right number of animals in the stables, so the problem often arises that not every animal has its own bedding area, which leads to major health problems. Such constructions are more expensive and require more space, which often discourages farmers from converting their stables.

In general, free-stall system provides higher animal welfare standards than tied rearing. However, there are many factors that can influence the welfare of the animals. As animal behaviour changes with age, the influence of a particular rearing system, and therefore the welfare status, may depend on the age of the animals. It may even turn out that at a certain stage of development (age) and under the influence of the environment, tied rearing is either comparable or even better in terms of animal welfare.

Considering the welfare of cattle, numerous articles have been published in recent decades dealing with the welfare of dairy cows (Knierim and Winckler, 2009; de Vries et al., 2013; Andreasen et al., 2013, 2014; Otten et al., 2020; Beaver et al., 2021; van Eerdenburg et al., 2021). In fattening cattle, the focus in the past has mainly been on the welfare of bulls (Gotardo et al., 2009; Kirchner et al. 2014a, 2014b; Schneider et al., 2020; Tarantola et al., 2020), while there is a lack of literature on the welfare of heifers. In addition, there are also no comparative studies on two different rearing systems (tied and free-stall) for heifers. Therefore, the aim of the present study was to evaluate the welfare status (using the Welfare Quality® protocol) of heifers in different rearing systems during entire rearing process in order to assess the temporal dynamics of animal welfare in the chosen systems.

We hypothesised that (i) animal welfare is generally higher in free-stall system than in tied rearing system, (ii) animal welfare varies according to the age of the animals, (iii) tied rearing system is more problematic in terms of animal welfare for younger animals that require more movement.

Materials and Methods

Study site and animals

The study took place on a local commercial farm near Rogaška Slatina (Slovenia). The farm is classified as a small farm with agricultural land in areas with limited possibilities for agricultural production (less-favoured areas). The farm has two types of rearing – tied and free-stall rearing. In each type, five heifers of different ages are reared at the same time, with the age of the animals being the same within a rearing system but varying between systems. The heifers are crosses of different breeds (Charolais, Limousin, Simmental). Tied rearing system means that the animal is tied permanently, thus combining rest, feeding and manure removal (three times a day). The barn is 6.5 m long and 4.50 m wide and the stalls are 1.95 m long and 0.8 m wide. The urine collection channel is 5.0 m × 0.3 m in size. There is a concrete crib in front where the animals are fed (6.0 m × 0.5 m × 0.3 m). The floor is made of concrete and straw is used for bedding. Drinkers are cup-type (one drinker for two heifers). The feed consists of hay, senage (most of it), fresh grass, maize silage, maize meal, barley and forage. In both systems, hay is fed in the morning, followed by haylage, and fresh grass in the evening (or maize silage, depending on the season). In contrast to confinement, free-stall rearing means that the animals are free to move around within a group pen. The farmer's job is only to feed the animals and check on them regularly. The barn is 4.80 m long and 3.90 m wide. The group pen is 3.90 m × 3.50 m in size. The floor is made of concrete slats. Two cup drinkers are used for watering. The feeding is carried out in a concrete crib (3.50 m × 0.55 m × 0.30 m). Small iron posts (10 in total) serve as a barrier for feeding. Both rearing systems have sufficient openings for light and air flow.

We monitored actual situation on the farm from the beginning of fattening to slaughter without any additional interventions in the daily breeding work and tasks. The approval of the ethics committee was therefore not required according to Directive 2010/63/EU (2010). On the farm, new animals/calves are usually brought in at an average age of around 7.5 months (280 kg live weight). The calves are purchased from Hungarian pastures and were therefore not bred in any of the systems practised on the farm. The heifers (whole groups at a time) are sold at the age of 24 to 27 months, depending on the market situation. During the study period, the animals were bought at 7.9 months of age thus sold at 27 months of age. Two repetitions were carried out thus a total of 20 animals were included in the study.

Welfare evaluation

The data collection took place over a period of 13 months. A total of seven assessments were carried out at intervals of around 3 months. All seasons were included. The welfare assessments began one week after the calves were purchased and ended one week before the heifers were sold.

The welfare status of the heifers was assessed using the Welfare quality protocol® (2009), which includes four main observation areas or principles (feeding, housing, health and behaviour). Each principle comprises two to four criteria (twelve in total), that are assessed by on-farm measures. To determine the suitability of each criterion and principle, a score between 0 and 100 is calculated, indicating the worst or best possible situation. The focus of the protocol is on the assessment of the individual animal. Most of the measurements and observations prescribed by the protocol are animal-based, although there are also some management-, farm- and pen-based measurements. The specificity of the protocol is also that good results/scores for one measure/criterion cannot compensate for poor results/scores for another measure/criterion (Welfare Quality®, 2009).

During the study, most of the data were obtained through direct observations and measurements of the animals in the barn. Only a small part of the information (availability of an outdoor run or pasture, dehorning and castration, mortality) was obtained from the breeder before the evaluation. Some data was also obtained from video recordings. The use of video recordings contributed to better monitoring of the animals. The recordings started at 8:20 am and lasted 120 minutes. The recordings were made simultaneously in both breeding systems using two telephones. Before recording began, the animals were fed dry feed (eliminating the influence of diet). A brief summary of the animal welfare assessments and the subsequent calculations of the welfare scores is given below, while a detailed description can be found in the Welfare Quality® protocol (2009).

Welfare measurements and observations

The first principle (feeding) consists of two criteria, i.e. the absence of a prolonged hunger, which is determined by measuring body condition (satisfactory, very lean), and the absence of prolonged thirst, which is assessed by the type of drinkers (e.g. cups), the cleanliness of the water points (clean, partially dirty, dirty) and the number of animals using the water points. The second principle (housing) is also divided into two criteria. Comfort at resting is made up of the time the animal needs to lie down and the cleanliness of the animal (the proportion of the body surface covered by pads or liquid dirt). The time is measured from the time the animal bends over and lowers the wrist to the time the animal pulls the front leg out from under the body (the average time for the animals assessed is reported). The second criterion, ease of movement, includes two measures: the dimensions of the cubicles in relation to the weight of the animal and access to outdoor areas/pastures. To assess the characteristics of the pen in relation to live weight, the dimensions of the pen, the number of animals in the pen and the weight of the animals are recorded. With regard to access to outdoor areas or pastures, it is indicated whether and to what extent (number of hours or days of access) access is available on the farm. The third principle relates to health and is divided into three criteria. The absence of injuries criterion records the frequency of lameness (percentage of lame animals) and skin lesions (percentage of animals with mild and severe lesions). The absence of disease criterion is assessed on the basis of mortality (deaths, euthanasia, emergency slaughter) and the occurrence of various symptoms (coughing, nasal and eye discharge, diarrhoea, obstructed breathing, bloated rumen). The third criterion for the health status is the absence of pain. Here we evaluate whether and how dehorning and castration are carried out on the farm. The fourth principle relates to behaviour, which consists of four criteria. Various aggressive and cohesive interactions are recorded to assess social behaviour. For negative interactions, pushing, shoving, chasing, fighting and chasing are recorded and expressed as the average number of aggressive behaviours per animal per hour. The same applies to cohesive behaviour, which includes social licking and horning. Under the criterion of other behaviours, access to pasture is assessed (number of days per year, number of hours per day). In order to measure good human-animal relationships, an avoidance test is carried out at the feeding site. At a distance of 3.5 metres from the animal, the assessor makes sure that the animal is attentive and then slowly approaches the animal, holding his arm at an angle of about 45 degrees. We record the percentage of animals that allow to be touched as well as the percentage of those that retreat at a distance of 50 cm, up to 100 cm or over 100 cm. Finally, we assess the animal's various emotional states using the Qualitative Behavioural Assessment (QBA) to assess 20 behavioural states and rate them on a scale from minimum to maximum.

Calculation of criterion and principle scores

The data collected for the animal unit are used to calculate criterion scores and these are further used to calculate the principle scores as described in the Welfare Quality® protocol (2009). To calculate the criteria scores different approaches are used, depending on the type of measurement (animal- or pen-/farm-based). For animal-based measurements, the data obtained in the stable is converted into an index, which is used to calculate the criterion score using the L-spline function. Such a method is used for the criteria of absence of hunger, comfort at resting, ease of movement, absence of injuries, expression of social behaviours, expression of other behaviours, human-animal relationship and emotional states. For the remaining two criteria, absence of thirst and absence of pain, where data are collected at group level, a decision tree is used to determine criterion scores. A criterion score reflects the farm's compliance with a particular criterion and is expressed on a value scale from 0 to 100, where 0 means the worst situation on a farm (it is assumed that there can be no further deterioration in welfare), 50 means a neutral situation (i.e. welfare is not bad, but not good either) and 100 means the best situation on a farm (it is assumed that there can be no further improvement in welfare).

To calculate the principle scores, the Choquet integral is used, which does not allow compensation between the criterion scores. As not all criteria are equally important, the weights are also required for this calculation. The weights are specified in the protocol. The principle scores also reflect the farm's compliance with certain principles and is also expressed on a value scale from 0 to 100, with 0 representing the worst and 100 the best possible situation.

Statistical analysis

The data analysis was carried out in SPSS. Basic statistics (medians, quartiles, frequencies, etc.) were calculated for all measurements, criterion scores and principle scores for both rearing systems. The non-parametric Mann-Whitney U test was used to compare the two rearing systems regardless of the age of the animals, as the variables were not normally distributed. The results are presented as a polar diagram separately for the criterion scores and the principle scores. In order to include the temporal component (age of the animals), scatter plots were created to show the dependence of the criterion and principle scores on the age of the heifers for both rearing systems. Using the analysis of covariance (Univariate General Linear Models) with the rearing system as a fixed factor and the age of the heifers as a covariate, trend lines were calculated and the slopes compared. In the case of a significant interaction between rearing system and age, the slopes of the trend lines for rearing systems differed significantly. There was no variability within the rearing system for the three protocol criteria, so no further analysis was performed for them.

Results and discussion

Welfare status of heifers in free-stall and tied rearing system

The comparison of criterion and principle scores for tied and free-stall rearing systems is shown in Figure 1, while the details of the animal welfare assessment are given in Table 1. The principle scores for feeding were 93.5 and 99.6 for tied and free-stall rearing systems, respectively, indicating that feeding as practiced on the farm can be in general considered appropriate. There were almost no heifers that were rated as lean (median of 99.4 in both systems). Considering the absence of thirst, the deviation from optimal conditions was only found in the tied rearing system (score of 93.0), where each confined animal had access to only one drinker (Table 1), whereas the protocol considers optimal conditions to exist when at least two drinkers are available to each animal. This is in line with a similar study on cows in tied housing, that also showed lower scores when only one drinker was available (Popescu et al., 2013). In a tied system, it is not possible to accommodate more drinkers due to limited space. However, both criterion scores were high and comparable in both rearing systems, and consequently no differences in the final score for the feeding principle were found between rearing systems.

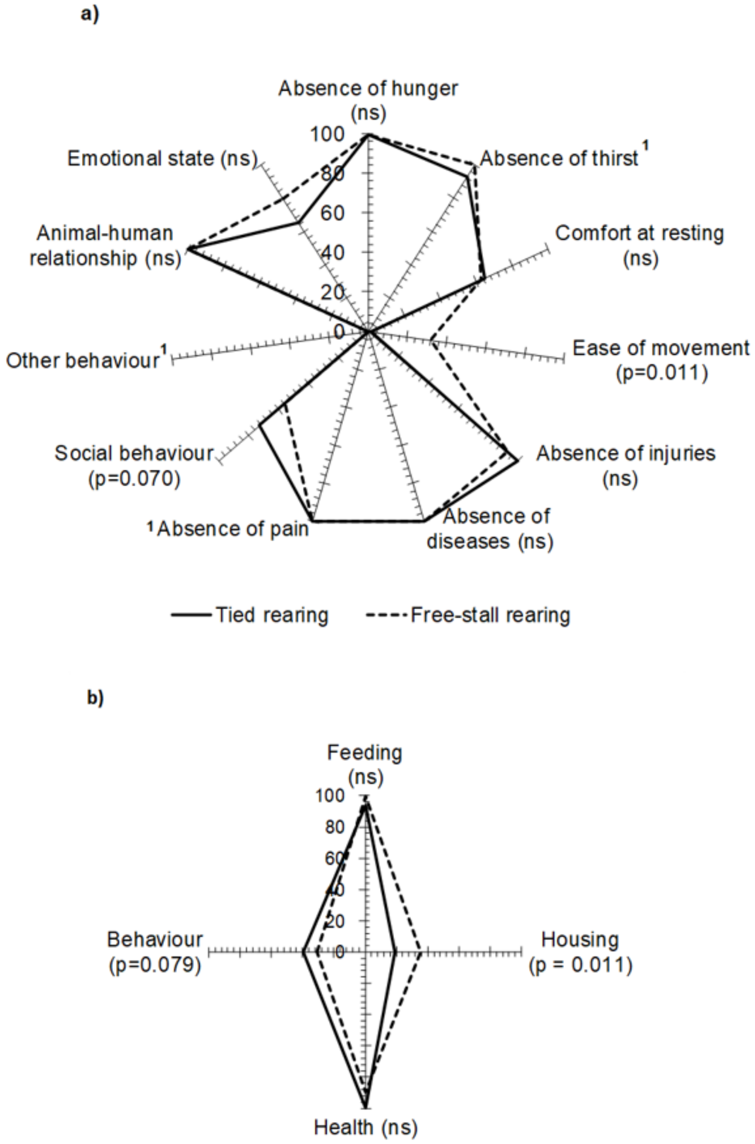
The final score for the housing principle was significantly higher in free-stall rearing system (35.7) than in tied system (19.1), although both were considerably lower compared to the feeding principle. The difference in the final score indicates differences between the systems. Based on video recordings, we were able to detect irregularities in heifer rearing (the heifers did not stretch their front leg at the end). The criterion of ease of movement was rated higher in the free-stall system than in the tied system (31.8 and 1.7 respectively). This criterion was evaluated on the basis of the space available for a 700 kg animal (2 m^2 being the minimum and 9 m^2 the maximum) and access to outdoor area. According to the results, the heifers in the free-stall system had more floor space (more space to move) (4.4 m^2 vs. 2.1 m^2). The results are in accordance with some previous studies, showing in most cases, there is more space in free-stall rearing (Popescu et al., 2013; Oehm et al., 2020; EFSA, 2023). However, the principle scores for housing were generally quite low for both tied and free-stall system, as the animals did not have the opportunity to graze in any of the rearing systems. Criterion scores for resting comfort, assessed by lying time and animal cleanliness, did not differ between rearing systems, with the percentage of heifers rated as dirty (20-30 %) and lying time of heifers (≈5 s) showing no differences between rearing systems.

The principle scores for health were high and did not differ significantly between the rearing systems (99.8 and 89.8 for tied and free-stall system, respectively). The median scores for absence of disease and absence of pain due to rearing interventions were 100 for both rearing systems. No diseases were detected in the barn during the study and no breeding interventions (castration, tail docking, etc.) were carried out. In both systems, integumentary changes occurred, but only in a mild form (e.g. hairless patches due to the stall equipment). Only one lame heifer was observed in the final fattening phase. The lameness can have various causes. In our case, we assume that it is due to the lack of space, which causes the animals to turn around more frequently (leading to hoof abrasion). Lameness is often caused by uneven and unsuitable ground. Fewer infectious and non-infectious foot diseases (white line disease, digitalin dermatitis, *E. coli*) were found in tie system than in free-stall system (Beaver et al., 2021).

In our study, the principle score for behaviour were generally quite low, but tended to be higher in tied system than in free-stall system (39.6 and 30.8, respectively). The reason for low scores was the fact that animals had no access to pasture (score 0.0). There were no significant differences between the rearing systems for the criterion human-animal relationship and emotional state. In both systems, the heifers showed a good human-animal relationship, as most of them allowed themselves to be touched by humans and showed no fear reactions (score 100 in both systems). Although positive emotional states prevailed in both rearing systems, the scores tended to be significantly higher in the free-stall than in the tied system (79.4 and 65.2, respectively). There were differences in the criterion of social behaviour. Contrary to our expectations, higher scores were achieved in tied system than in free-stall rearing. The results showed a higher frequency of cohesive and especially agonistic interactions between the heifers in tied rearing system (72.7 for tied and 55.7 for free-stall). In tied housing, the heifers have less space than in free-stall housing, so they spend their time (when they would otherwise be moving around) grooming each other. Studies have shown that this is due to the restrictions on movement (Popescu et al., 2013). Here, the protocol has been shown to have shortcomings that would need to be addressed to achieve a more realistic outcome. For example, there are some omissions in the equations and in the final scores. As mentioned earlier, tied rearing scores are better than free-stall in the behavioural principle due to the time spent on grooming. In the case where no access to outdoor or grazing is possible, the score is 0, which has a significant impact on the final score. It should also be emphasised that the evaluation of QBA is highly subjective, even if the subjectivity is somewhat mitigated by the wide range of different emotional states. Also, agonistic and socio-positive behaviours in animals can change multiple times daily (fluctuation in emotional state) as found by Kirchner et al. (2014b). Nevertheless, some heifers gain weight faster than others, even when eating the same ration, so scoring body condition of calves is not always a relevant indicator.

According to the results, the most important factor in cattle rearing is the space available for the animals. In case of tied system, the animals had the same rearing area during the whole fattening period, i.e. long stalls of 1.95 m × 0.8 m. In free-stall system, an area of 2.5 m^2/animal is recommended for young cattle (220 kg). During this period, an area of 2.7 m^2/animal was provided. The problem occurred in the finishing phase, in which the recommended area per animal is 4.2 m^2. To ensure an optimal floor area, the barn would need to be enlarged by 35%. This is not a restriction, but a recommendation (IURŽ, 2014). If the optimal free-stall area had been achieved, this would have resulted in a higher score. The movement of the animals and a larger floor area contribute to better welfare, but could lower profit (Ahmed et al., 2020).

In summary, tied rearing system scored slightly better in the behaviour principle (a higher number of cohesive behaviours), while free-stall scored significantly better in terms of good housing, due to the larger exercise area, larger floor area and good feeding (where the animals have more drinkers). Otherwise, there were no major differences between the rearing systems. Low scores for a specific criteria are characteristic of both smaller and larger farms (the same score regardless of the size of the farm) (Gottardo et al., 2009).



ns – not significant (p≤0.05), [1]no variation within one or both rearing systems (no statistical test applied)

Figure 1: Comparison of criterion scores (a) and principle scores (b) in tied and free-stall rearing

Table 1: Results of assessment using Welfare Quality protocol and calculation of scores[1]

Principle	Criterion	Measurement/ observation	Results		Criterion score		Principle score	
			Tied rearing	Free-stall rearing	Tied rearing	Free-stall rearing	Tied rearing	Free-stall rearing
Feeding	Absence of hunger	% of lean cows	0.0 [0.0–0.0]	0.0 [0.0–20.0]	99.4 [99.4–99.4]	99.4 [5.7–99.4]	93.5 [93.5–93.5]	99.6 [30.2–99.6]
	Absence of thirst	No. of drinking places, no. of cows per drinker, cleanness of drinkers	One drinker per 2 heifers, access to one drinker only, cleanness OK	Two drinkers per 5 heifers, access to two drinkers, cleanness OK	93.0 [93.0–93.0]	100.0 [100.0–100.0]		
Housing	Comfort around resting	Time needed to lie down (seconds)	4.6 [4.0–5.3]	4.9 [4.4–5.5]	64.7 [47.0–86.0]	62.5 [44.7–91.3]	19.1 [11.3–26.4]	35.7 [30.9–79.53]
		Cleanness of the animals (% of dirty cows)	20.0 [0.0–40.0]	20.0 [0.0–40.0]				
	Ease of movement	Pen features according to the weight of animals (m^2/700 kg live weight)	2.3 [1.7–3.2]	4.0 [3.2–6.2]	1.7 [0.0–15.2)	31.8 [15.7–75.4]		
		Access to outdoor loafing area or pasture	No access	No access				
Health	Absence of injuries	Lameness (% of lame cows)	No lame cows	No lame cows	99.8 [86.7–99.9]	93.0 [75.4–99.8]	99.8 [89.8–100.0]	89.8 [68.9–94.6]
		Integument alterations (% of cows with mild and severe alterations)	Mild: 0.0 [0.0–40.0] No severe cases	Mild: 20.0 [0.0–60.0] No severe cases				
	Absence of disease	Mortality and different symptoms (nasal, ocular discharge, coughing, hampered respiration, diarrhoea, bloated rumen)	No warning or alarm threshold exceeded	No warning or alarm threshold exceeded	100.0 [100.0–100.0]	100.0 [100.0–100.0]		
	Absence of pain	Dehorning – method and use of medicines	Not applied	Not applied	100.0 [100.0–100.0]	100.0 [100.0–100.0]		
Behaviour	Expression of social behaviour	Mean number of agonistic behaviours per cows per hour	0.9 [0.1–1.2]	2.6 [2.3–5.0]	72.7 [68.5–95.1]	55.7 [21.6–67.3]	39.6 [35.9–41.6]	30.8 [29.7–37.8]
		Mean number of cohesive behaviours per cows per hour	3.3 [1.8–3.7]	2.3 [1.9–4.0]				
	Expression of other behaviours	Access to pasture	No access	No access	0.0 [0.0–0.0]	0.0 [0.0–0.0]		
	Human-animal relationship	Avoidance distance (% of animals): 0 cm (can be touched)	100.0 [100.0–100.0]	100.0 [60.0–100.0]	100.0 [100.0–100.0]	100.0 [77.9–100.0]		
		< 50 cm	0.0 [0.0–0.0]	0.0 [0.0–0.0]				
		50–100 m	0.0 [0.0–0.0]	0.0 [0.0–0.0]				
		> 100 cm	0.0 [0.0–0.0]	0.0 [0.0–0.0]				
	Emotional state	Qualitative behaviour assessment			65.2 [63.2–77.0]	79.4 [70.2–89.2]		

[1] The results are presented as median [first quartile–third quartile].

Temporal dynamics of heifer welfare in free-stall and tied system

A comparison of the criteria and principles in relation to the age of the heifers in tied and free-stall system is shown in Figure 2. With regard to the feeding principle, there were no differences between the two rearing systems in terms of temporal dynamics. Overall, welfare increased with age in both systems. In line with the general assessment, the criterion scores for the absence of hunger showed a similar temporal trend: they were lowest when the animals were moved into the study pens and reached maximum values in

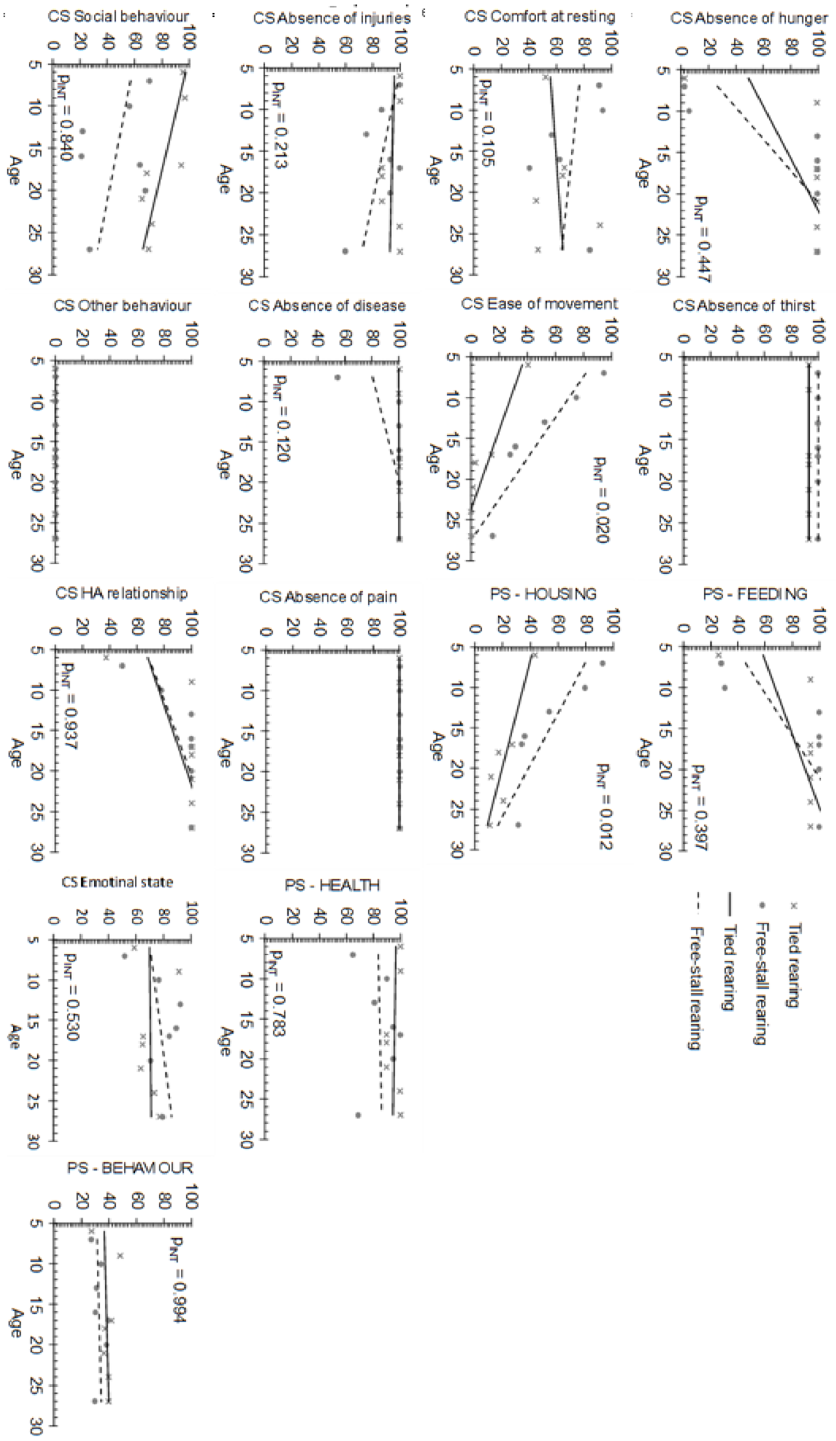


Figure 2: Temporal dynamics of welfare assessments in tied and free-stall system

CS – criterion score; PS – principle score; HA – human-animal; p_{INT} – p value for interaction Rearing system × Age both systems by the third assessment. However, the lower initial scores were probably the ongoing effect of the conditions from which the animals came and not of the system to which the animals were moved in. These results suggest that the first observation should be carried out later (2 weeks after the animals were housed), which is in agreement with recommendation for ruminant research in nutritional study (Machado et al., 2016). The water supply was constant within the rearing system during the observation period (no variation), and consequently scores were at the same level throughout the fattening period (93 for the tied system and 100 for the free-stall system). The number of drinkers did not change during fattening and the score remained the same, as observed also in the study of Popescu et al. (2013).

In contrast to the feeding principle, significant differences were found in the temporal dynamics of the rearing principle between the rearing systems studied. At the beginning of the fattening period, the scores for the free-stall system were considerably higher. In both systems, the animal welfare scores decreased with age (larger animals and thus less space for movement), but more rapidly in the free-stall system. Younger animals require less space and therefore have more freedom of movement (better scores). As body weight increases, space becomes limited, which was the reason for the lower scores for this criterion.

No differences in the temporal dynamics of welfare scores were found for the health principle. In both systems, the scores were very high and mainly constant over the entire observation period. The slightly lower score on the health criteria for the absence of disease in young animals could also be due to transportation. The slight decrease in the criterion score for the absence of injuries in the free-stall system is a consequence of the occurrence of lameness, which increases with age. In this system, the animals have more opportunity to move and express their emotions, instincts, etc., which could lead to leg injuries and consequently lameness. The percentage of lameness would be even higher in males when they reach sexual maturity (Lunstra et al., 19878). It is worth noting that there was a difference in the flooring, because free-stall animals were kept on concrete slats, whereas animals in tied rearing had bedding (straw). Straw is less aggressive for legs and consequently there are fewer injuries and health problems (Tuyttens, 2005). In the study of Eldahshan et al. (2023), it was found that free-stall rearing heifers were more resistant due to the higher leukocyte counts.

There were no differences in the final score for behaviour. The criterion scores for social behaviour were higher in the tied system, as the heifers spent more time grooming each other, but decreased over time in both systems. As the animals had no opportunity to graze, the criterion score for other behaviour was lowest in both systems. Criterion scores for human-animal interactions were initially lower (again, this is a long-lasting effect of the previous rearing conditions, namely free-stall pasture system with little human interaction), but rose quite rapidly to the highest score in both rearing systems, showing that calves that have come to the farm from pasture quickly become accustomed to being close to the breeder. It was also found by Masebo et al. (2023) that the immediate assessment of animal welfare after transportation leads to lower scores due to stress (new environment and feed, mixing of animals). The free-stall system provides better conditions for the expression of positive emotional states, as criterion scores increased over time. Another study also argues that free-stall housing is better suited for expressing emotions (Eldahshan et al., 2023). As a result of the temporal dynamics of all four criterion scores, the principle score for behaviour was similar in both rearing systems and constant during the observation period.

In summary, the temporal dynamics of the welfare scores did not differ between tied and free-stall rearing system except for the housing principle. Free-stall housing in general offered better conditions. However, with increasing age of the heifers, the criterion and principle scores decreased over time in both systems, but more rapidly in the free-stall system. The heifers gained weight and, as a result, there was less space available for normal lying and exercise.

Conclusion

Although animal welfare parameters did not differ in all aspects between rearing systems, animal welfare was generally higher in free-stall systems mainly de to higher scores for housing conditions. Our results confirmed tied stall less suitable for young animals in particular. If both systems are present in the breeding facilities, breeders should pay particular attention to ensure that young animals are primarily kept in free-stall system.

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Dobro počutje telic različnih starosti v dveh sistemih reje

IZVLEČEK

Dobro počutje domačih živali, ki je premisa sodobne živinoreje, je močno odvisno od sistema reje. V govedoreji je sistem vezane reje še vedno zelo pogost, čeprav zaradi omejene možnosti gibanja velja za manj primernega. Alternativa temu sistemu je kot minimalni standard predlagana hlevska prosta reja, ki živalim omogoča prosto gibanje na omejenem območju. Čeprav se zdi samoumevno, da je prosta reja boljša od vezane, še vedno ni raziskav o tem, ali to velja za vse kategorije in starosti govedi. Namen raziskave je bil primerjati dobro počutje telic v vezani in prosti reji v obdobju pitanja pri različnih starostih. Raziskava je vključevala pet telic v vsakem sistemu na ponovitev (skupaj 20 živali). Počutje telic smo ocenili z uporabo protokola Welfare Quality®, ki vključuje oceno štirih opazovalnih področij: krmljenje, bivalni pogoji, zdravje in obnašanje. Meritve in opazovanja živali in hleva se pretvorijo/preračunajo v ocene dobrega počutja od 0 (neprimerno stanje) do 100 (optimalno stanje). Ocenjevanja so bila izvedena sedemkrat v obdobju pitanja (6–27 mesecev starosti) v trimesečnih intervalih. Rezultati so pokazali značilne razlike v ocenah dobrega počutja živali med vezano in prosto rejo le pri bivalnih pogojih ter tendenco razlik pri obnašanju. Pri obeh področjih so bile ocene razmeroma nizke v primerjavi s področjem krmljenja in zdravja živali, kjer med sistemoma reje ni bilo ugotovljenih razlik. Tudi časovna dinamika ocen meril in področij je bila značilno različna le pri bivalnih pogojih. Pri obeh sistemih reje so se vrednosti s starostjo zmanjševale. V sistemu proste reje so bile ocene pri mladih živalih skoraj optimalne (> 90), a so se s starostjo hitro znižale, v sistemu vezane reje pa ocene niso bile optimalne že pri mladih živalih (≈ 40). Rezultati kažejo, da je vezana reja manj primerna z vidika dobrega počutja živali zlasti za mlade živali. Zaradi tega je priporočljivo, da se mlade živali, če sta v vzrejnih objektih na voljo oba sistema, nastanijo v ograde s prosto rejo.

Ključne besede: dobro počutje živali, govedo, telice, sistem reje, protokol ocenjevanja

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Comparison of Slovenian Traditional Plum Materials with Genetic Resources from the Slovenian Plant Gene Bank

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ABSTRACT

The Slovenian Plant Gene Bank (SPGB) of the Faculty of Agriculture and Life Sciences houses approximately 250 accessions of stone fruit, with most of the material belonging to the species *Prunus domestica* L. and *Prunus cerasifera* Ehrh. The main objectives of this study using a set of 11 SSR primers were: 1. to determine the genetic structure of the traditional Slovenian *in situ* plum material in comparison to the *ex situ* the SPGB collection; 2. to identify unique material among the *in situ* collected accessions; 3. to gain insight into the genetic relationship between the two studied species. The genetic structure of 60 plum samples was analyzed using Principal Coordinate Analysis (PCoA) and Bayesian model-based analysis. PCoA separated the *P. cerasifera* and *P. domestica* accessions, while Bayesian model-based analysis revealed that many accessions of *P. domestica* and *P. cerasifera* shared a common ancestral history. The *ex situ* material showed greater genetic diversity as it was distributed over more populations than the *in situ* material. Promising *in situ* genotypes, especially from the Prekmurje and Lower Styria, were identified as valuable additions to enrich the existing collection.

Keywords: plum, *Prunus domestica* L., *Prunus cerasifera* Ehrh., SSR markers, genetic structure

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INTRODUCTION

Germplasm collections serve as an important reservoir of plant genetic resources as well as a source of diversity and are essential for successful crop improvement (Butac, 2020). As a result of careful collection, conservation and evaluation, these collections provide a valuable source of genes (e.g., wild genotypes, landraces, local populations, clones and lines bred from indigenous plant materials and ecotypes) that can be used in breeding programs or as cultivars with high tolerance to abiotic and biotic stresses, suitable for sustainable agricultural practices (Blazek, 2007; Butac et al., 2010; Dey et al., 2016).

The Slovenian Plant Gene Bank (SPGB) of the Faculty of Agriculture and Life Sciences is part of the Slovenian Plant Gene Bank Program (SRGB), which aims to preserve, evaluate, regenerate and conserve indigenous Slovenian germplasm, including local ecotypes, populations and landraces of agricultural, medicinal and aromatic plants, as well as forest trees and other woody plants from Slovenian forests (Šiško, 2016). It was established in 2007 on an area of approximately 3 ha and is located in an isolated location according to FAO standards next to the Botanical Garden of the University of Maribor (Pivola). It is a permanent collection plantation intended both for the storage of accessions and for evaluation of accessions. Part of the collection, including vines, is located in the Meranovo viticulture center (Limbuš). The germplasm collection includes accessions from the following genera: *Prunus*, *Rubus* and *Vitis*. After 17 years of work on the collection, there are currently approximately 250 accessions belonging to stone fruit (e.g., plums, sweet and sour cherries, apricots, peaches, and almonds). Of these, around 170 plum accessions belong to the *Prunus domestica* L. and *Prunus cerasifera* Ehrh. species (Šiško, 2018 and unpublished data from the SPGB collection).

The European plum, *P. domestica* ($2n = 6x = 48$), and the myrobalan plum, *P. cerasifera* ($2n = 2x = 16$) are species which belong to a group of European plums (Hartmann et al., 2009; Neumuller, 2011). The first species is used worldwide in fruit production due to the versatility of the fruit use (fresh, dried

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or processed) (Milošević et al., 2023). It is also known to form different pomological groups (based on fruit use and morphological characteristics such as fruit shapes and color), including large-fruited European plums, prunes, egg plums, greengages, mirabelles, damsons, bullaces and St. Julien plums (Zhebentyayeva et al., 2019; Gaši et al., 2020). The use of fresh and dried fruits of *P. cerasifera* is limited to traditional use in Western Asia (Hanelt, 2001; Okie and Hancock, 2008), while in Europe, myrobalan is often used for processing (e.g., jams and chutneys) and for the spirit production (Topp et al., 2012). In addition, *P. cerasifera* is resilient species and adapted to a wide range of rural and urban areas (forest edges, open woodlands, along roads and rivers, abandoned orchards, around farm buildings, gardens and parks) (Hartmann et al., 2009; Popescu and Caudullo, 2016). It is widely used as an ornamental tree and as an important rootstock for other *Prunus* species (e.g., plum, peach, apricot and almond) (Sancin, 1988; Sedaghathoor et al., 2009; Das, 2011).

The use of plant genetic resources, in particular when they are not present in dedicated conservation centers, is often limited by insufficient information, such as material identification, phenotypic and genetic diversity and pomological data (Milošević and Milošević, 2018). Comprehensive information on the phenotypic and genotypic characteristics of the material and various preservation strategies are crucial for the conservation process (Ramanatha and Hodgkin, 2002). Traditionally, plum material is characterized and identified based on morphological traits (Martínez-Gómez et al., 2005). However, this method of distinguishing genotypes has limitations, as some traits are unreliable (e.g., variations in growing conditions, plant age, and phenological stage) (Mehdi et al., 2012). To identify plant genetic relationships, structure and diversity, molecular marker technology has developed in recent decades to replace or complement morphological markers (Soriano, 2020).

As part of a broader research endeavor (Ternjak et al., 2023), the main objective of this study was to determine the genetic structure of the traditional Slovenian *in situ* plum material in comparison to the *ex situ* material of SPGB collection, using SSR molecular markers. The focus was on identifying unique material among the accessions collected *in situ* that could be used to enrich the collection. In addition, we wanted to gain insight into the genetic relationship between the two studied species (*P. domestica* and *P. cerasifera*).

MATERIALS AND METHODS

Plant material

The accessions examined in this research are part of a broader study investigating the genetic diversity and structure of three plum species: *P. domestica*, *P. cerasifera*, and *P. spinosa* (Ternjak et al., 2023). This paper focuses specifically on the analyzes and comparison of plum material belonging to *P. domestica* and *P. cerasifera* collected in Slovenia (*in situ* and *ex situ*).

From 2018 to 2019, young and healthy leaves were collected from 60 plum accessions. Roughly half of the material (29 accessions) belonged to *P. domestica* species and 31 accessions to *P. cerasifera*. Sixteen accessions were collected *ex situ* in SPGB, while 44 accessions were collected *in situ* from different regions in Slovenia (Lower Styria, Prekmurje, Upper Carniola and Istria). Each sample was documented, and for the *in situ* accessions, the exact location of the tree was determined using latitude and longitude coordinates (WGS84 system). For the *ex situ* accessions, the location of the collection site was recorded together with the data on the origin of the material. Table 1 lists the data of the collected material, including name, species, status, origin and other passport data.

Table 1: The data of the 60 genotypes (29 accessions of *Prunus domestica* L. and 31 accessions of *Prunus cerasifera* Ehrh.), including name, species, origin and other passport data as well as the membership values to the populations were analyzed with STRUCTURE (Pritchard et al., 2000)

Sample No.	Name	Species	Accession ID	Conservation type	Origin of the material	Structure analysis	
						K = 2	K = 7
37	Plum_green_37	*P. domestica*	/	*ex situ* SPGB*	Brdce, Vojnik, Lower Styria	K2	6
38	Plum_38	*P. cerasifera*	/	*in situ*	Črnc, Brežice, Lower Styria	K1	2
44	Bluish_plum_44	*P. domestica*	/	*in situ*	Lendavske gorice, Prekmurje	K1	2
45	Bluish_plum_45	*P. domestica*	/	*in situ*	Lendavske gorice, Prekmurje	K1	2
46	Bluish_plum_46	*P. domestica*	/	*in situ*	Lendavske gorice, Prekmurje	K1	2

Sample No.	Name	Species	Accession ID	Conservation type	Origin of the material	Structure analysis	
						K = 2	K = 7
51	Plum_51	*P. cerasifera*	6356	*ex situ* SPGB*	Črnc, Brežice, Lower Styria	K1	2
52	Plum_52	*P. domestica*	6085	*ex situ* SPGB*	Črnc, Brežice, Lower Styria	K1	2
55	Plum_55	*P. cerasifera*	6372	*ex situ* SPGB*	Trnje pri Brežicah, Lower Styria	K1	2
58	Plum_58	*P. cerasifera*	6370	*ex situ* SPGB*	Trnje pri Brežicah, Lower Styria	K1	Admixed
59	Plum_59	*P. cerasifera*	6391	*ex situ* SPGB*	Trnje pri Brežicah, Lower Styria	K1	4
60	Plum_60	*P. cerasifera*	6371	*ex situ* SPGB*	Trnje pri Brežicah, Lower Styria	K1	3
61	Plum_61	*P. cerasifera*	/	*ex situ* SPGB*	Brezina, Brežice, Lower Styria	K1	3
62	Plum_62	*P. cerasifera*	6392	*ex situ* SPGB*	Trnje pri Brežicah, Lower Styria	K1	3
63	Plum_63	*P. cerasifera*	6394	*ex situ* SPGB*	Trnje pri Brežicah, Lower Styria	K1	1
67	Bluish_plum_67	*P. domestica*	3576	*ex situ* SPGB*	Maribor, Lower Styria	K1	1
76	Plum_76	*P. cerasifera*	6350	*ex situ* SPGB*	Črnc, Brežice, Lower Styria	K1	1
79	Common_prune_Bistrica_79	*P. domestica*	6317	*ex situ* SPGB*	NA	K1	1
82	Plum_82	*P. cerasifera*	6347	*ex situ* SPGB*	Črnc, Brežice, Lower Styria	K1	1
84	Common_prune_Bistrica_84	*P. domestica*	6416	*ex situ* SPGB*	Sromlje, Brežice, Lower Styria	K1	1
92	Bluish_plum_92	*P. domestica*	/	*in situ*	Maribor, Lower Styria	K1	1
101	Bluish_plum_101	*P. domestica*	/	*in situ*	Orehova vas, Lower Styria	K1	1
102	Bluish_plum_102	*P. domestica*	/	*in situ*	Orehova vas, Lower Styria	K1	1
103	Bluish_plum_103	*P. domestica*	/	*in situ*	Orehova vas, Lower Styria	K1	1
104	Bluish_plum_104	*P. domestica*	/	*in situ*	Orehova vas, Lower Styria	K1	1
105	Bluish_plum_105	*P. domestica*	/	*in situ*	Orehova vas, Lower Styria	K1	1
106	Bluish_plum_106	*P. domestica*	/	*in situ*	Orehova vas, Lower Styria	K1	1
107	Common_prune_107	*P. domestica*	/	*in situ*	Orehova vas, Lower Styria	K1	1
108	Common_prune_108	*P. domestica*	/	*in situ*	Orehova vas, Lower Styria	K1	Admixed
109	Common_prune_109	*P. domestica*	/	*in situ*	Orehova vas, Lower Styria	K2	5
110	Common_prune_110	*P. domestica*	/	*in situ*	Orehova vas, Lower Styria	K2	5
111	Plum_111	*P. cerasifera*	/	*in situ*	Orehova vas, Lower Styria	K2	5
112	Bluish_plum_112	*P. domestica*	/	*in situ*	Maribor, Lower Styria	K2	5
113	Bluish_plum_113	*P. domestica*	/	*in situ*	Limbuš, Lower Styria	K2	5
114	Bluish_plum_114	*P. domestica*	/	*in situ*	Limbuš, Lower Styria	K2	5
116	Bluish_plum_116	*P. domestica*		*in situ*	Lovrenc na Pohorju, Lower Styria	K2	5

Sample No.	Name	Species	Accession ID	Conservation type	Origin of the material	Structure analysis	
						K = 2	K = 7
117	Common_prune_117	*P. domestica*	/	*in situ*	Miklavž na Dravskem polju, Lower Styria	K2	5
118	Plum_118	*P. cerasifera*	/	*in situ*	Maribor, Lower Styria	K2	5
119	Plum_119	*P. cerasifera*	/	*in situ*	Maribor, Lower Styria	K2	Admixed
120	Plum_120	*P. cerasifera*	/	*in situ*	Maribor, Lower Styria	K2	5
121	Plum_121	*P. cerasifera*	/	*in situ*	Maribor, Lower Styria	K2	5
122	Plum_122	*P. cerasifera*	/	*in situ*	Maribor, Lower Styria	K2	5
129	Plum_yellow_129	*P. cerasifera*	/	*in situ*	Koper, Istria	K2	5
130	Plum_bluish_130	*P. cerasifera*	/	*in situ*	Koper, Istria	K2	5
131	Plum_red_131	*P. cerasifera*	/	*in situ*	Koper, Istria	K2	5
132	Plum_yellow_132	*P. cerasifera*	/	*in situ*	Koper, Istria	K2	5
133	Plum_yellow_133	*P. cerasifera*	/	*in situ*	Izola, Istria	K2	5
134	Plum_violet_134	*P. cerasifera*	/	*in situ*	Strunjan, Istria	K2	5
135	Plum_red_135	*P. cerasifera*	/	*in situ*	Vas Dragonja, Istria	K2	5
136	Plum_bluish_136	*P. cerasifera*	/	*in situ*	Krkavče, Istria	K2	5
137	Myrobalan_137	*P. cerasifera*	/	*in situ*	Kasaze, Lower Styria	K2	5
138	Myrobalan_138	*P. cerasifera*	/	*in situ*	Kasaze, Lower Styria	K2	5
139	Myrobalan_139	*P. cerasifera*	/	*in situ*	Kasaze, Lower Styria	K2	5
140	Mirabelle_140	*P. domestica*	/	*in situ*	Kasaze, Lower Styria	K2	5
145	Bluish_plum_145	*P. domestica*	/	*in situ*	Ruše, Lower Styria	2	5
146	Bluish_plum_146	*P. domestica*	/	*in situ*	Ruše, Lower Styria	2	5
147	Plum_147	*P. cerasifera*	/	*ex situ*	Črnc, Brežice, Lower Styria	2	5
153	Spindel_plum_153	*P. domestica*	/	*in situ*	Rašica, Upper Carniola	2	7
155	Plum_yellow_155	*P. cerasifera*	/	*in situ*	Maribor, Lower Styria	2	5
156	Plum_red_156	*P. cerasifera*	/	*in situ*	Maribor, Lower Styria	2	5
189	Bluish_plum_189	*P. domestica*	/	*in situ*	Zakot, Brežice, Lower Styria	2	5

* SPGB (Slovenian Plant Gene Bank)

Molecular analyzes

DNA isolation and molecular markers analyzes

Total genomic DNA was extracted from the young leaf material following the CTAB protocol described by Doyle and Doyle (1987), with some modifications. Two separate extractions per sample were performed. The DNA concentration of each sample was estimated using a fluorimeter (Hoefer DQ 300, California, USA). The quality was also checked on a 3% agarose gel by electrophoresis (Bio-Rad, California, USA), and the products were visualized under UV light.

All studied accessions were analyzed using a set of eleven 11 SSR primer pairs: UDP96-005, BPPCT034, EMPAS12, UCD-CH17, EMPAS06, EMPAS11, EMPAS14, BPPCT014, BPPCT025, CPSCT026 and CPPCT006, which were developed on different *Prunus* species. Information on marker selection, polymerase chain reaction (PCR) amplification procedures and fragment size analysis was previously published by Ternjak et al. (2023).

Data analyzes

The genetic structure among the studied accessions was analyzed using the Principal Coordinate Analysis (PCoA) and complemented with the Bayesian model-based clustering method. For the PCoA calculations, the microsatellite allele data were converted into a binary matrix. Dissimilarities were calculated with Sokal and Michener index and transformed into Euclidean distances using the 0.5 power transformation. Using DARwin 6.0.21 software (Perrier and Jacquemoud-Collet, 2006), each accession was assigned to a location in a two-dimensional space and the figure was constructed. The STRUCTURE V2.3.4 software package (Pritchard et al., 2000) was used to perform the Bayesian model-based clustering method. Ternjak et al. (2023) have already presented a detailed description of the settings and construction of the bar plots. The most relevant parameter K (number of populations) for the analyzed data was determined by calculating ΔK according to the method described by Evanno et al. (2005). This calculation was performed with the Structure Harvester V0.6.94 application (Earl and von Holdt, 2012). The individuals with a membership coefficient (ql) > 0.9 were assigned to a specific population, and those with a threshold value below the estimated membership were considered admixed. The STRUCTURE 2.3.4 software (Pritchard et al., 2000) was also used to compute the average distances (expected heterozygosity) between individuals within the same population.

RESULTS AND DISCUSSION

Principal Coordinate Analysis provided insight into the distribution of different plum groups and allowed us to obtain a global representation of diversity. In the analysis, the examined material was divided according to species and when considering the conservation type (*in situ* and *ex situ*) the material was distributed over the entire graph (Fig. 1).

The accessions of *P. cerasifera* were located on the left side of the figure and formed a denser arrangement in space, while the accessions of *P. domestica* were located on the right side of the figure. The only exception was the *ex situ* accession Plum 52 on the left side of the figure, which clustered slightly closer to the *P. cerasifera* species. Although the study by Ternjak et al. (2023) considered Plum 52 to be *P. domestica,* as the flow cytometry results confirmed that the accession was hexaploid, the analyzed SSR profiles also showed similar behavior to other *P. domestica* accessions. However, morphological observations also revealed a similarity with the *P. cerasifera* species. In addition, genetic diversity assessment using three universal cpDNA primers showed that Plum 52 belonged to haplotype H4, which was shared by *P. domestica* and *P. cerasifera* (Ternjak et al., 2023). Possibly, accession Plum 52 is an example of a putative hybrid origin, which is not surprising as *P. domestica* can easily hybridize with *P. cerasifera* (Topp et al., 2012). The *P. domestica* material was more scattered on the right side of the figure, forming different clusters. On the upper right side of the figure, two clusters of the landrace Bluish plum could be observed. The Bluish plum accessions mostly collected *in situ* (except Bluish plum 67) were positioned according to their origin, with a larger cluster of Bluish plums from the Styria region and a smaller cluster of only three Bluish plum accessions (44,45 and 46) from the Prekmurje region. On the lower left side of the figure, four accessions were highlighted, material slightly different from the other clusters: Bluish plum (189), which was also collected in the Styria region, but is genetically distinct and was separated from the two Bluish plum clusters; Plum green (37), a small-fruited landrace with green color, yellow-oval-fruited landrace Spindel plum (153) and the accession Mirabelle 140, which belongs to the mirabelle pomological group. Mirabelles are a specialty of the Lorraine region in France, sweet small-fruited plums that are predominantly yellow to orange, often with red spots (Gaši et al., 2020). The PCoA analysis also identified a pomological group of common prunes which included *in situ* and *ex situ* material and clustered in the lowest part of the figure.

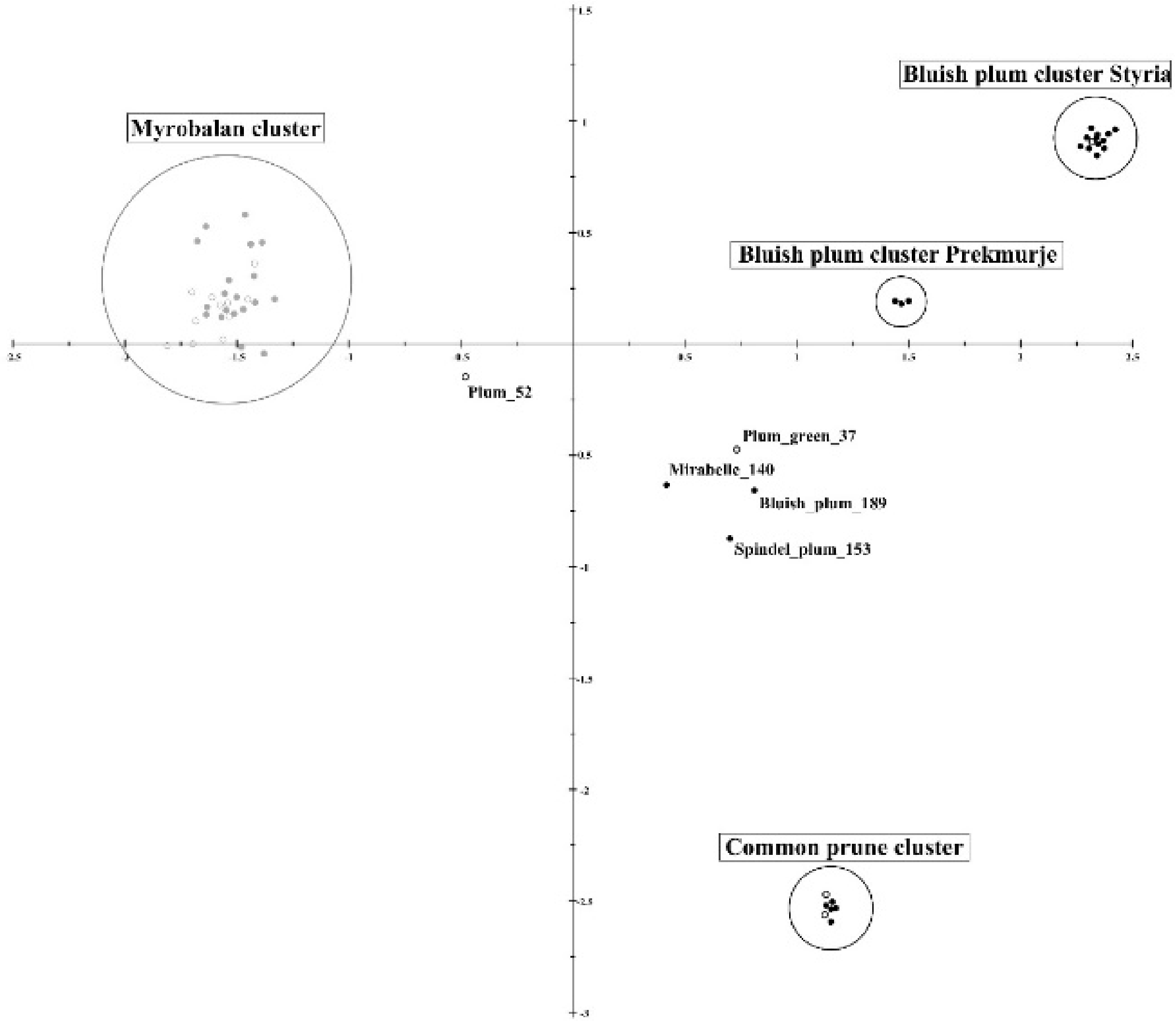

Figure 1: Principal Coordinate Analysis (PCoA) based on polymorphism at 11 SSR loci of the 60 plum genotypes. The accessions of *P. cerasifera* Ehrh. (Myrobalan cluster, grey color) formed a dense cluster, whereas the material of *P. domestica* L. (black color) is more scattered and consists of different clusters. The accessions marked with a full dot represent *in situ* material, while the accessions with an empty dot are *ex situ* material

Bayesian model-based clustering analysis revealed the structural patterns and divided the analyzed material into ancestral populations. The maximum value for ΔK was K = 2 (975.15), dividing the material into two populations (Fig. 2, Fig. 3 and Table 2). Population K1 (bar plots in black color) accounted for 27 genotypes and consisted of 13 *in situ* and 14 *ex situ* accessions (Table 1). Population K2 (bar plots in grey color) accounted for 33 genotypes, mostly belonging to the *in situ* material (31), with two *ex situ* accessions (Table 1). This collected material has the potential to increase and enrich the SPGB genetic resources collection. Both populations were comprised of various accessions belonging to both studied species and different pomological groups. For the K = 2, no material was considered admixed.

Table 2: Table summarizing the results using Evanno et al. (2005) method, output of Structure Harvester V0.6.94 application (Earl and von Holdt, 2012)

# K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	10	-10143.72000	0.85739	NA	NA	NA
2	10	-7461.45000	2.21071	2682.27000	2155.77000	975.14990
3	10	-6934.95000	94.39210	526.50000	186.26000	1.97326
4	10	-6594.71000	223.41714	340.24000	36.47000	0.16324
5	10	-6290.94000	210.95657	303.77000	1477.68000	7.00466
6	10	-7464.85000	4418.45931	-1173.91000	2612.48000	0.59126
7	10	-6026.28000	4.16034	1438.57000	1386.09000	333.16733
8	10	-5973.80000	5.99574	52.48000	32.35000	5.39550
9	10	-5953.67000	18.74923	20.13000	125.85000	6.71228
10	10	-6059.39000	25.61642	-105.72000	NA	NA

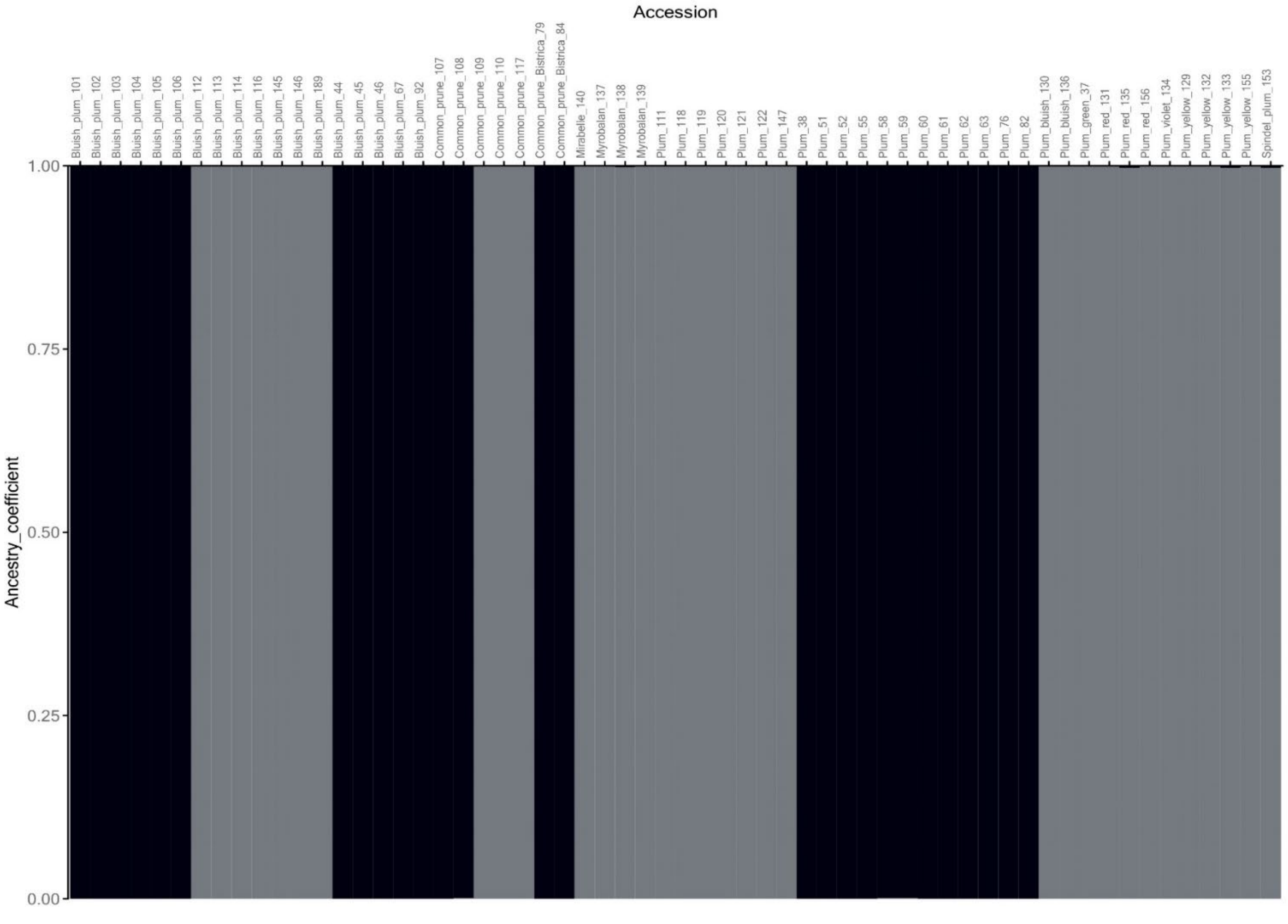

Figure 2: Bar plot of the results of the Bayesian model-based clustering (K = 2) for 60 plum genotypes. Population K1 accessions are shown in black and Population K2 accessions are shown in grey. No accessions were admixed

In terms of allelic variation within the two populations, STRUCTURE software revealed that the mean distances between individuals were the greatest in Population K1 (0.7410), while the distances were smaller in Population K2 (0.5213) (Table 3).

Table 3: Average distances (expected heterozygosity) between individuals in the same population

Population K1:	0.7410
Population K2:	0.5213

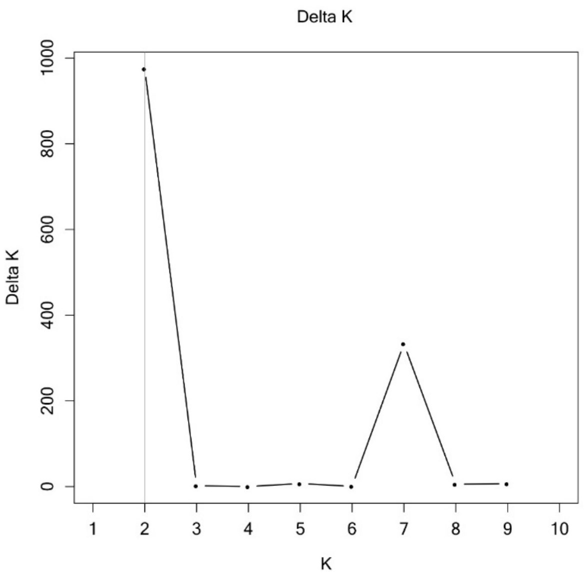

Figure 3: Graphical method, as in Evanno et al. (2005), allowing the detection of the number of groups K for the 60 plum genotypes using ΔK

The Evanno criterion, used to evaluate the genetic structure showed a weaker signal for K = 7 (333.17), dividing the material into seven populations (Fig. 4, Fig. 3 and Table 2). Population K1 split into four sub-clusters (Populations 1-4), while Population K2 split into three sub-clusters (Populations 5-7) (Table 1). Most accessions (14) belonging to Population 1 (black) maintained the same profile or remained unchanged from the original Population K1 (Plum: 63, 76, 82; Bluish plum: 67, 92, 101, 102, 103, 104, 105, 106; Common prune: 79, 84 and 107). Seven accessions, Bluish plums: 44, 45, 46 separated and formed a new Population 2 (light grey) that also included accessions Plum: 38, 51, 52 and 55, while Population 3 (grey with black dots) contained three accessions (Plum: 60, 61 and 62). Population 4 (white) consisted of a single accession (Plum 59). Most accessions (30) were assigned to Population 5 (dark grey) and had the same profile or remained unchanged from the original Population K2. The other two populations contained only one accession, namely Population 6 (white with black stripes), Plum green (37) and Population 7 (black with white dots), Spindel plum (153). In the remaining three admixed accessions, the largest part of their genome 0.7 for Plum 58, 0.87 for Plum 119 and 0.7 for Common prune 108, was associated with populations 3, 6 and 7, respectively.

The results of the STRUCTURE software for K = 7 have also highlighted some accessions or groups that stood out for their originality. For some of them, results were consistent with the PCoA analysis, e.g., Plum green (37) and Spindel plum (153). For the other accessions, Bayesian model-based clustering analysis showed that they belonged to a new ancestral population, that was not shown in previous analysis. For example, Plum 59 from Population 4, or accessions from the Population 3.

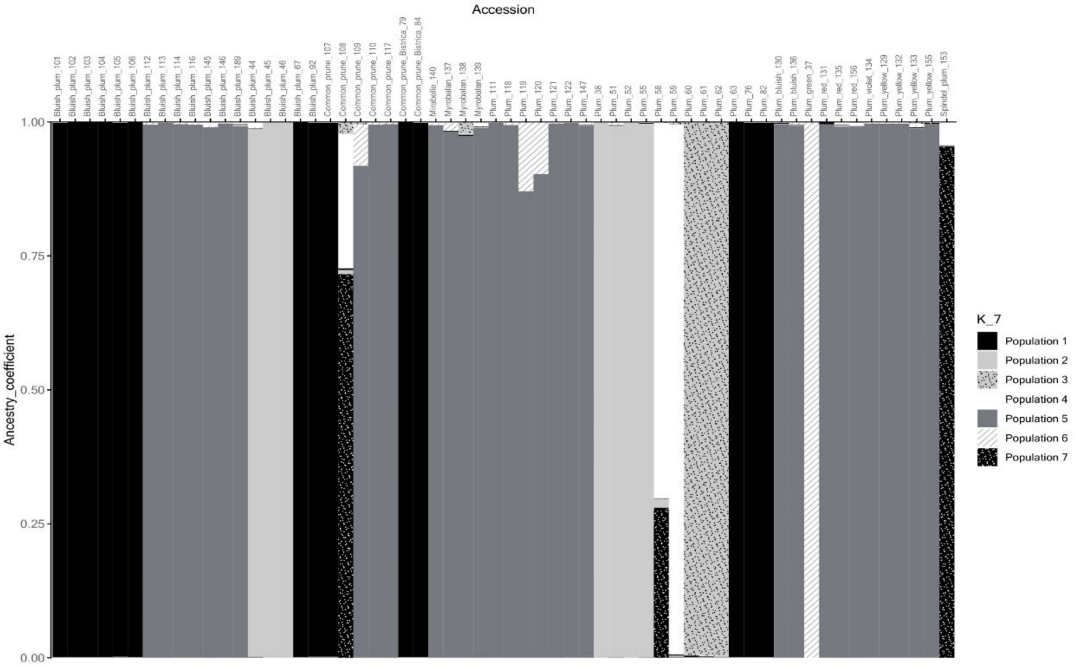

Figure 4: Bar plot of the results of the Bayesian model-based clustering analysis (K = 7) for 60 plum genotypes. Population 1 accessions are shown in black, Population 2 accessions are shown in light grey, Population 3 accessions are shown in grey with black dots, Population 4 accessions are shown in white, Population 5 accessions are shown in dark grey, Population 6 accessions are shown in white with black stripes and Population 7 accessions are shown in black with white dots. Three accessions were admixed.

When comparing *in situ* and *ex situ* material at K = 7, the first was distributed among four ancestral populations (1, 2, 5 and 7), while the second was distributed among six populations (1, 2, 3, 4, 5 and 6) and thus showed higher genetic diversity. This was expected, as the material from the SPGB collection had been selected for its high diversity. Nevertheless, we found promising material among the accessions collected *in situ* that could be included to enrich the existing collection. For example, Bluish plum genotypes originating from the Prekmurje region and belonging to Population 2 (accessions 44, 45 and 46), as well as Bluish plum genotypes belonging to Population 5 and originating from Lower Styria, but both different from Bluish plum material in the current SPGB collection. In addition, we also discovered common prune genotypes belonging to Population 5, which also differed from the existing collection. Another example was already mentioned, Spindel plum (153) which was pointed out as a unique material by the both analyzes.

When we studied the structure of *P. domestica* and *P. cerasifera* together with other wild relatives such as *P. spinosa*, the Bayesian model-based clustering analysis revealed *P. domestica* and *P. cerasifera* as independent groups (Ternjak et al., 2023). Interestingly, the present study, which focused exclusively on the analysis of *P. domestica* and *P. cerasifera* material, showed that many accessions of the two species belonged to the same population and thus share ancestral history. This is supported too by the results of genetic diversity assessment studies using chloroplast DNA markers reported by Bortiri et al. (2009), Reales et al. (2010), Horvath et al. (2011) and Ternjak et al. (2023), in which *P. domestica* and *P. cerasifera* clustered together, suggesting that *P. cerasifera* may have contributed to the maternal chloroplast DNA of *P. domestica*.

CONCLUSIONS

We aimed to determine the genetic structure of traditional Slovenian *in situ* plum material in comparison to the *ex situ* material of the SPGB collection, using SSR molecular markers. The combined results provided valuable insights into the genetic diversity and structure of the Slovenian plum genetic resources. PCoA clearly separated the *P. cerasifera* and *P. domestica* accessions, with *P. cerasifera* forming a dense cluster and the *P. domestica* accessions being more scattered and consisting of different clusters. An exception was accession Plum 52, which, although classified as *P. domestica*, genetic markers suggesting a hybrid origin with *P. cerasifera*. An exception was accession Plum 52, which, although classified as *P. domestica*, clustered near *P. cerasifera*. The combined results of the different analyzes based on genetic markers therefore suggest a hybrid origin with *P. cerasifera*. The *ex situ* material showed higher genetic diversity and was distributed among more populations compared to the *in situ* material, reflecting the selection for high diversity that was made when accessions were introduced into the SPGB collection. Nonetheless, promising *in situ* accessions, particularly from the Prekmurje and Lower Styria regions, were identified as valuable additions to enrich the existing collection. Certain accessions, such as Plum green (37) and Spindel plum (153), were highlighted as unique by both PCoA and Bayesian model-based clustering analysis. Bluish plum populations from different regions and common prune genotypes were identified as distinct from the ones in existing collections, suggesting potential new additions to the Slovenian germplasm collection. The analyzes revealed that many accessions of *P. domestica* and *P. cerasifera* share a common ancestral history. This finding is consistent with previous studies using chloroplast DNA markers, which also suggested that *P. cerasifera* may have contributed to the maternal chloroplast DNA of *P. domestica*. Overall, this study demonstrated the utility of integrating molecular markers and advanced clustering methods to uncover the complex genetic relationships and diversity within plum species to aid future breeding and conservation efforts.

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Primerjava slovenskih tradicionalnih genotipov sliv z genskimi viri iz Slovenske rastlinske genske banke

IZVLEČEK

Rastlinska genska banka Fakultete za kmetijstvo in biosistemske vede hrani poleg drugih vrst tudi okrog 250 akcesij koščičarjev, pri čemer večina materiala pripada vrstama *Prunus domestica* L. in *Prunus cerasifera* Ehrh. Z uporabo 11 mikrosatelitskih lokusov smo v raziskavi želeli: 1. oceniti genetsko strukturo slovenskih tradicionalnih genotipov sliv nabranih *in situ* v primerjavi z *ex situ* materialom iz kolekcije genske banke koščičarjev; 2. prepoznati edinstven material med *in situ* nabranimi akcesijami; 3. raziskati genetske odnose med dvema proučevanima vrstama. Genetska struktura 60 genotipov sliv je bila analizirana s pomočjo Principalne koordinatne analize (PCoA) ter Bayesove analize. PCoA analiza je razdelila akcesije glede na pripadnost preučevanima vrstama (*P. cerasifera* oz. *P. domestica*), medtem ko je Bayesova analiza pokazala, da si številne akcesije tako iz vrste *P. domestica*, kot *P. cerasifera* delijo pripadnost znotraj specifične populacije. Material nabran *ex situ* je pokazal večjo genetsko raznolikost, saj je bil razdeljen na več populacij v primerjavi z materialom nabranim *in situ*. Med slednjim smo identificirali unikatne genotipe sliv, predvsem iz Prekmurja in Štajerske, ki bi jih bilo smiselno vključiti v obstoječo kolekcijo rastlinske genske banke.

Ključne besede: sliva, *Prunus domestica* L., *Prunus cerasifera* Ehrh., mikrosatelitski markerji, genetska struktura

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The Adverse Impacts of a Single Exposure to the Fungicide Picoxystrobin during the Larval Stage on Africanized *Apis mellifera*

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ABSTRACT

Pesticide use remains a problem in agriculture, contaminating natural ecosystems and affecting bees. Fungicides have been widely used worldwide, and honey bees can bring contaminated pollen and nectar to the colony, exposing the larvae. Studies on larval exposure to fungicides are still rare. Therefore, this work aimed to evaluate the effects of larval exposure to the fungicide picoxystrobin on biological parameters and cellular stress in the fat body. The larvae were single exposure on the fourth day (D4) to picoxystrobin at concentrations of 5 ng a.i./μL (PCX5), 45 ng a.i./μL (PCX45), 135 ng a.i./μL (PCX135), and 400 ng a.i./μL (PCX400). The effects on larval and pupal mortality, pupation rate, and emergence were evaluated. Additionally, cellular stress in the fat body was assessed in newly emerged bees. Exposure to PCX400 increased larval mortality by 26% and reduced the emergence of adult bees. The other concentrations did not affect larval and pupal mortality, or pupation and emergence rates. A cytotoxicity effect was observed in newly emerged bees from PCX400, indicated by positive immunolabeling of HSP70. Thus, a single exposure to picoxystrobin can impair larval development, induce a cellular stress response, and may interfere with colony dynamics.

Keywords: development, honey bee, non-target organism, strobilurin, toxicity

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INTRODUCTION

Brazil is the largest country in South America and is recognized as an essential food supplier in global agriculture. It produces a substantial amount of the food consumed worldwide (Calil and Ribera, 2019). The country has vast arable lands, abundant resources, and a favorable climate, making the cultivating of many crops feasible (Martinelli et al., 2010). The most cultivated crops include soybeans, sugarcane, maize, coffee, oranges, rice, cotton, beans, and tobacco (Bordonal et al., 2018; Toloi et al., 2021; Valdes, 2022), contributing to Brazil achieving US$ 125 billion in agricultural export value in 2021 (Valdes, 2022). Even with great importance in agriculture, Brazil still faces internal challenges such as inefficient agricultural sub-sectors, land distribution inequality, environmental concerns, and the need for sustainable practices (Martinelli et al., 2010). Among these challenges, the use of pesticides has raised concerns among researchers regarding the damage to human health and the risk to the environment, as the country is one of the top consumers of pesticides worldwide (Tang et al., 2022).

Many studies have warned about the harmful effects of pesticides on human health and the potential risks of related diseases (Paumgartten, 2020; Islam et al., 2021; Lopes-Ferreira et al., 2022). Insecticides, herbicides, and fungicides are the most frequently used pesticides in Brazil (Lopes-Ferreira et al., 2022), and their usage has also been associated with terrestrial and aquatic contamination (Daam et al., 2019; Fernandes et al., 2020; Guarda et al., 2020; Brovini et al., 2021). Additionally, the impact of pesticide use extends to pollinators, e.g., bees, posing significant threats to ecosystems and biodiversity (Goulson et al., 2015; Sgolastra et

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al., 2020), and efforts must be made to mitigate this.

The global bee population demonstrates high diversity, with over 20,000 described species (Orr et al., 2021), and Brazil significantly contributes to this richness with more than 3,000 bee species (Silveira et al., 2002). However, Brazil's most well-known bee species is the poly-hybrid Africanized *Apis mellifera* (non-native), resulting from crossbreeding European and African subspecies (Sheppard et al., 1991). These managed bees have a high defense capability, remain active in foraging for extended periods, and are more efficient in resource collection compared to European subspecies (Winston and Katz, 1982; Malaspina and Stort, 1987). Furthermore, *A. mellifera* serves as a model for pesticide regulation in Brazil (Cham et al., 2017). Pesticide use in the country, however, is closely linked to the weakness and collapse of Africanized *A. mellifera* colonies (Pires et al., 2016).

Among pesticides, fungicides are widely used worldwide (Gikas et al., 2022). Nevertheless, studies on the effects of fungicides on non-target organisms receive less attention compared to insecticides and herbicides (Wood and Goulson, 2017; Zubrod et al., 2019). This is concerning, as field concentrations of fungicide residues may exceed levels considered safe by regulatory agencies (Rondeau and Raine, 2022). Cullen et al. (2019) suggest that further research is needed, employing diverse approaches, various species, and a wide range of compounds to reduce the current knowledge gap.

Picoxystrobin ($C_{18}H_{16}F_3NO_4$) is a fungicide from the strobilurin group; it acts by inhibiting the mitochondrial respiration (halting the production of ATP) of fungi (Bartlett et al., 2002). Nevertheless, previous studies have revealed that picoxystrobin can also be harmful to amphibians (Li et al., 2016), fish (Jia et al., 2018), soil animals (Schnug et al., 2015), and bees (Domingues et al., 2017; Batista et al., 2020). Adult workers of Africanized *A. mellifera* exposed continuously to the fungicide picoxystrobin had their lifespan reduced by 51.76%, along with an overload of the hepato-nephrocitic system (Domingues et al., 2017). Cytotoxic effects of picoxystrobin exposure after 24, 48, 72, and 96 hours were also observed in the midgut of Africanized *A. mellifera*, which can affect the individual performance of bees and may impact the colony as a whole (Batista et al., 2020).

In the environment, bees can be exposed to picoxystrobin and other strobilurins through direct spray application or by residues found in pollen, nectar, and water that they collect (Pettis et al., 2013; Simon-Delso et al., 2014; Samarghandi et al., 2017; Rondeau and Raine, 2022). This exposure may pose a potential risk to honey bee larvae as well. Additionally, picoxystrobin has been detected in crops visited by *A. mellifera* (Rondeau and Raine, 2022).

Benuszak et al. (2017) highlighted the need to use larvae in studies on honey bees' exposure to pesticides. From this perspective, it is essential to study honey bee larvae, as the ingestion of fungicide residues can cause stress, disturb their post-embryonic development, and potentially weaken the colony. Furthermore, this stress can activate cellular defense mechanisms and induce the expression of heat shock proteins (HSPs) (Tkáčová and Angelovičová, 2012). According to Silva et al. (2006), HSPs are valuable cellular biomarkers for pesticide exposure.

Based on the information mentioned above and considering that research assessing the effects of fungicides on *A. mellifera* larvae is still scarce compared to studies on insecticides (Aupinel et al., 2007; Silva et al., 2015; Tavares et al., 2015; Dai et al., 2017; Friol et al., 2017; Tavares et al., 2019; Tesovnik et al., 2020; Begna et al., 2023; Carneiro et al., 2023; Ke et al., 2023), although adverse effects have been reported (Simon-Delso et al., 2017; Tadei et al., 2019; Tadei et al., 2020; Zhang et al., 2020; Domingues et al., 2021). The present study aimed to evaluate the effects of larval exposure to the active ingredient of fungicide picoxystrobin through biological parameters. The response to cellular stress in the fat body was evaluated by detection of HSP70. It is crucial to determine whether exposure to picoxystrobin adversely affects larval development and induces stress responses, as this can help predict possible negative effects on honey bee colonies and their ecological and economic roles. In addition, it can guide regulatory decisions on fungicide use in agriculture and support strategies to protect bees and other pollinators.

MATERIALS AND METHODS

Colonies of Africanized *A. mellifera*

The honey bee larvae used in the present study were sampled from three different healthy colonies at an apiary located in the rural area of Piedade, São Paulo State (23°37′5.506"S, 47°29′7.926"W). The physiological status of the colonies were known, and no chemical treatments was applied to manage the colonies before or during the study period. In Brazil, research on invertebrates does not require animal ethics approval.

Chemicals: fungicide picoxystrobin and insecticide dimethoate

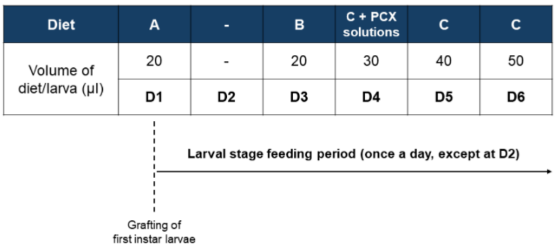
The picoxystrobin Pestanal® analytical standard (CAS number 117428-22-5, ≥ 98.0%) and dimethoate Pestanal® analytical standard (CAS number 1219794-81-6, ≥ 95.0%) were used for the larval toxicity tests. These standards were purchased from the Pestanal® product line, a registered trademark of Merck KGaA, Darmstadt, Germany.

Honey bee larval toxicity test, single exposure to picoxystrobin

The methodology followed the Organisation for Economic Co-operation and Development No. 237 protocol (OECD, 2013). Initially, a brood comb from each of the three colonies was collected and taken to the "Laboratory of Ecotoxicology and Environmental Integrity Analysis (LEIA)" at the "Federal University of São Carlos (UFScar)" in Sorocaba, São Paulo State, where the larval bioassay was performed.

The first instar larvae were individually transferred to sterilized polystyrene grafting cells (1 x 1 x 1 cm) with a wetted paintbrush (number 0), with each cell holding 20 µl of the standardized artificial diet A. The diet was composed of 50% by weight of fresh royal jelly and 50% by weight of an aqueous solution containing D-(+)-glucose (≥99.5%), D-(−)-fructose (≥99%), and yeast extract, as described by Aupinel et al. (2005). The polystyrene grafting cells were placed in cell culture plates (48 wells), each containing a piece of cotton soaked in 500 µl of sterilization solution (0.2% w/v methylbenzethonium chloride) enhanced with 15% w/v glycerol at the bottom of the wells. The plates containing the larvae were then placed into an acrylic desiccator cabinet (Thermo Scientific™ Nalgene™, 178 x 305 x 305 mm), where beakers containing a saturated solution of potassium sulphate (K_2SO_4) were also added to maintain humidity. The acrylic desiccator cabinet was kept in an incubator set at 34±2 °C, with a relative humidity of 90±5 %, under dark conditions.

The larvae were fed once a day until the sixth day (D6), and the diets and volumes were adapted at different stages of development, as described by Aupinel et al. (2005). On the fourth day of the experiment (D4), the larvae were single exposed to picoxystrobin concentrations (Fig. 1). First, a stock solution of picoxystrobin (1000 ng a.i./µL) was prepared in autoclaved distilled water (60%) and acetone (40%) and diluted serially to obtain the working concentrations of 5, 45, 135, and 400 ng a.i./µL. Since the fungicide picoxystrobin is not completely soluble in water (3.1 mg/L at 20 °C), acetone was used as an organic solvent, and a solvent control (CAC) was also added following the protocol described by the OECD No. 237 (2013), not exceeding 5% of the final diet volume (1.5 µL of acetone for a diet volume of 30 µL on D4). The control group (CTL) received only the larval diet without adding additional chemicals. Dimethoate (DMT) was used as a toxic reference chemical (8.8±0.5 µg a.i./larva) to ensure the reliability of the experiment (OECD, 2013).



Diet	A	-	B	C + PCX solutions	C	C
Volume of diet/larva (µl)	20	-	20	30	40	50
	D1	D2	D3	D4	D5	D6

D – day; PCX – picoxystrobin

Figure 1: Schematic representation of the larval stage feeding period adapted from OECD No. 237 protocol for larval toxicity test, single exposure (OECD, 2013). The diets A, B, and C were based on Aupinel et al. (2005).

On the day of the single exposure (D4), the honey bee larvae were divided into the following experimental groups: picoxystrobin at 5 ng a.i./µL (PCX5), picoxystrobin at 45 ng a.i./µL (PCX45), picoxystrobin at 135 ng a.i./µL (PCX135), picoxystrobin at 400 ng a.i./µL (PCX400), control (CTL), solvent control (CAC), and dimethoate positive control (DMT). Fourteen honey bee larvae were used from each of the three selected healthy colonies per experimental group. This resulted in 42 larvae per experimental group, meeting the OECD No. 237 (OECD, 2013) requirement of a minimum of 36 honey bee larvae per group. The specific concentrations used in this study were based on preliminary studies conducted in the LEIA at UFSCar.

Evaluation of the biological effects of single exposure

After pesticide exposure on the fourth day (D4), the larval mortality rate of all experimental groups was monitored for up to 72 hours (D5-D7). The pupation mortality and pupation rates were monitored from the eighth to the fifteenth day (D8-D15), and the cumulative emergence rate was recorded on the twenty-second day (D22).

Immunofluorescence "*in totum*" for HSP70 detection

Three newly emerged bees (up to 48 hours old) that had been exposed to picoxystrobin during the larval stage were sampled from CTL, CAC, and PCX400 groups. They were then anesthetized by exposure to a low temperature (4 °C) for one minute and dissected in a sodium chloride (0.9%) using a stereomicroscope (Leica EZ4 HD) to remove the dorsal vessel along with the parietal fat body.

The dissected organs from all selected groups were placed individually on positively charged silanized slides (ImmunoSlide, EasyPath), where drops of the fixative solution (paraformaldehyde 4% in phosphate-buffered saline (PBS), 0.1 mol L^{1}, pH 7.4) were added for 24 hours at 4 °C and covered with a plastic coverslip to spread the solution. The entire procedure was carried out in a black incubation tray for immunohistochemistry (EasyPath). After the fixation period, the slides containing the organs were washed in PBS and then incubated for 10 minutes in PBS with 0.05% Tween® 20 (pH 7.4). The organs were subsequently permeabilized using a solution of 0.5% Triton X-100 in PBS for 30 minutes, followed by three washes in PBS with 0.05% Tween® 20, with a five-minute incubation during the final wash. Nonspecific antigenic sites were blocked using PBS with 0.05% Tween® 20 and 3% bovine serum albumin (BSA) solution for one hour at room temperature. The slides with organs were then washed three times in PBS with 0.05% Tween® 20 and incubated with a primary antibody solution (monoclonal anti-heat shock protein 70, antibody produced in mouse, Clone BRM-22, H5147 - Sigma-Aldrich™), diluted 1:100, for five days in a black incubation tray in the fridge at 4 °C. After incubation with the primary antibody, the slides containing the organs were washed in PBS with 0.05% Tween® 20 for 30 minutes. Incubation was then carried out with the secondary antibody (rabbit anti-mouse IgG (H+L) cross-adsorbed, conjugated with Alexa Fluor™ 488, Invitrogen - Thermo Fisher Scientific, A-11059), diluted 1:100, for one hour at room temperature. Following this incubation, the slides were washed three times in PBS buffer and mounted with an aqueous fluorescence mounting medium (Dako) using glass coverslips. Two negative reaction controls were also performed (without primary and secondary antibodies).

Immunofluorescence analyses were conducted to localize HSP70 using a laser scanning confocal microscope (LEICA TCS-SP8) with Leica Application Suite X software (LAS X, version 3.5.5), following the configurations described by Domingues et al. (2017). Three slides, each prepared from a single bee, were analyzed per group.

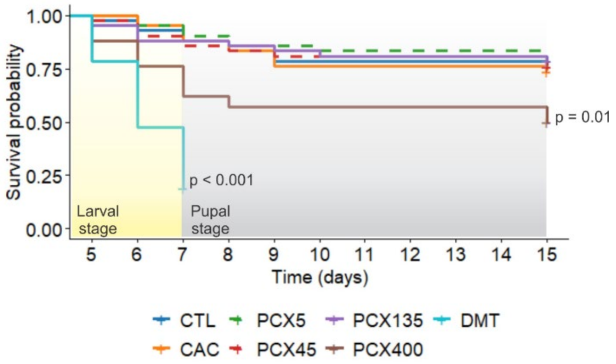
Statistical analysis

Data analysis was performed using R software, version 4.2.2. Survival data from larval and pupal stages were analyzed using the Log-rank test from the "survival" package (Therneau, 2021). The occurrence of bee pupation and emergence for each individual was computed up to the fifteenth day (D15) and twenty-second day (D22), respectively. Then, the pupation and emergence events were analyzed using generalized linear models with quasibinomial and binomial distributions, with the experimental groups as independent variables. The goodness of fit of the statistical models to the data was checked by half-normal plots (Moral et al., 2017). The pupation and emergence proportions of each experimental group were contrasted with the control group using estimation of effect size analysis with 5,000 resamples from the "dabestr" package (version 2023.9.12, Ho et al., 2019) generating Cohen's h and p-value from a two-sided permutation t-test.

RESULTS

Biological effects of a single exposure to picoxystrobin

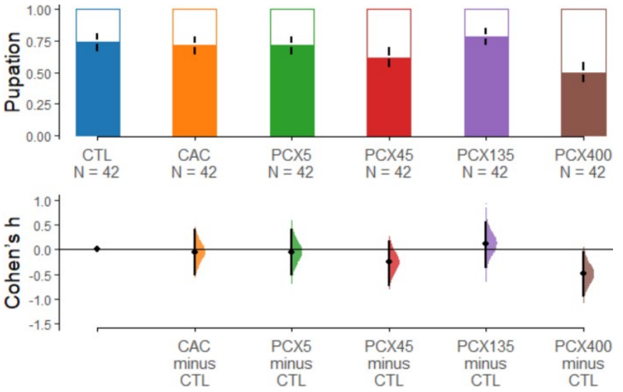
The larval exposure to pesticides, considering DMT, increased the mortality of Africanized honey bees during the larval stage ($\chi^2=109$, df=6, $p<0.001$), but did not influence the survival probability during the pupal stage ($\chi^2=1.3$, df=5, p=0.9), as shown in Figure 2. During the larval stage, larvae from the CAC, PCX5, PCX45, and PCX135 groups showed similar survival probabilities to the CTL group ($p>0.91$). Exposure to PCX400 increased larval mortality by 26% compared to the CTL group (p=0.013).. The highest larval mortality was observed in the DMT group, which reduced survival probability by 69% compared to the CTL group ($p<0.001$), validating the larval toxicity test according to the OECD No. 237 protocol (OECD, 2013).



CTL – Control; CAC – solvent control; PCX5 – picoxystrobin at 5 ng a.i./μL; PCX45 – picoxystrobin at 45 ng a.i./μL; PCX135 – picoxystrobin at 135 ng a.i./μL; PCX400 – picoxystrobin at 400 ng a.i./μL; DMT – dimethoate as a positive control. n = 42 honey bee larvae per experimental group.

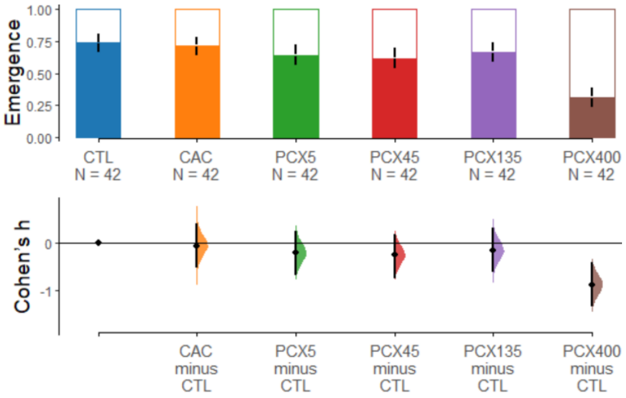
Figure 2: Survival probability of Africanized honey bees during the larval and pupal stages after single pesticide exposure.

The pupation rate was not impaired by picoxystrobin exposure (Quasibinomial GLM, $\chi^2=9.98$, df=5, p=0.087). Compared to pupae from the CTL group, pupae from all groups exhibited a weak Cohen's h with values ranging from -0.5 to 0.2 (Fig. 3). However, a negative influence of picoxystrobin exposure was observed on the emergence rate (Binomial GLM, $\chi^2=21.311$, df=5, p=0.0007), with a reduction in the number of newly emerged adults when exposed to PCX400 (p=0.0001), as depicted in Figure 4.



CTL – Control; CAC – solvent control; PCX5 – picoxystrobin at 5 ng a.i./μL; PCX45 – picoxystrobin at 45 ng a.i./μL; PCX135 – picoxystrobin at 135 ng a.i./μL; PCX400 – picoxystrobin at 400 ng a.i./μL.

Figure 3: Proportion of Africanized honey bees that reached the pupal stage after larval exposure to picoxystrobin. The inferior axis displays 95% effect size bootstraps of Cohen's h values obtained by comparing the experimental groups with the control group (indicated by the horizontal black line).

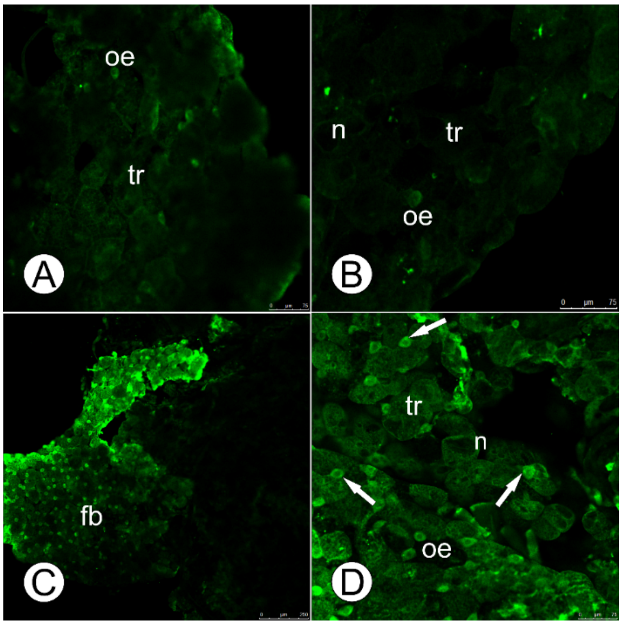


CTL – Control; CAC – solvent control; PCX5 – picoxystrobin at 5 ng a.i./μL; PCX45 – picoxystrobin at 45 ng a.i./μL; PCX135 – picoxystrobin at 135 ng a.i./μL; PCX400 – picoxystrobin at 400 ng a.i./μL.

Figure 4: Proportion of Africanized honey bees that reached the adult stage after larval exposure to picoxystrobin. The inferior axis displays 95% effect size bootstraps of Cohen's h values obtained by comparing the experimental groups with the control group (indicated by the horizontal black line).

Detection of HSP70 in the fat body

Figure 5 shows the cellular stress response following exposure to picoxystrobin, as evidenced by the detection of HSP70 in the fat body of newly emerged Africanized *A. mellifera*. The oenocytes and trophocytes of bees from the CTL and CAC groups exhibited similar response patterns, characterized by either basal levels or the absence of immunolabeling of HSP70 (Fig. 5A and Fig. 5B). Furthermore, HSP70 labeling was not observed in the cell nuclei. Regarding the fat body of bees from the PCX400 group, positively immunolabeled regions were observed (Fig. 5C). These regions were not identified in the CTL and CAC groups. The response pattern of oenocytes was also altered in bees from the PCX400 group, with evidence of labeled HSP70 in the cytoplasm, specifically in the perinuclear region (Fig. 5D), a feature not observed in the CTL and CAC groups.



A – Control (CTL); B – solvent control (CAC); C – picoxystrobin at 400 ng a.i./μL (PCX400); fb – fat body; n – nuclei; oe – oenocyte; tr – trophocyte; white arrow – positive labeling of HSP70; n – three newly emerged honey bees per experimental group.

Figure 5: Detection of HSP70 in the fat body of newly emerged Africanized honey bees exposed to the fungicide picoxystrobin during the larval stage.

DISCUSSION

The results presented in this study highlight that larval exposure to the fungicide picoxystrobin can increase larval mortality and reduce bee emergence, even if only at the highest concentration (400 ng a.i./μL). This finding is concerning, as bees may be exposed to high concentrations of fungicide through pollen, nectar, and water (Pettis et al., 2013; Zubrod et al., 2019; Zioga et al., 2020). According to Thompson et al. (2014), the toxicity of fungicides may increase in a dose-dependent manner due to ingestion by honey bees. In that regard, studies focusing on the prolonged contact of larvae and adult bees with fungicides are needed to better understand disruptions in developmental processes and physiological responses linked to cellular stress.

Regarding the other picoxystrobin concentrations used in this study, neither larval mortality rates nor post-embryonic development were significantly affected.. The absence of adverse effects on these parameters was similarly observed in studies performed with the active ingredient pyraclostrobin (Tadei et al., 2019; Domingues et al., 2021) and its commercial formulation (Tadei et al., 2020). Fungicide pyraclostrobin belongs to the strobilurin chemical class, similar to picoxystrobin (Bartlett et al., 2002). On the other hand, when fungicides were combined with insecticides, larvae were less likely to survive to adulthood (Wade et al., 2019).

In addition to the observed effects on the development parameters in the PCX400 group, oenocytes from the parietal fat body of newly emerged bees exhibited positive immunolabeling for HSP70, indicating a cellular stress response. Similar findings were described in the intestine after larval exposure to the fungicide pyraclostrobin, where positive labeling for HSP70 was observed (Tadei et al., 2020). According to Malaspina and Silva-Zacarin (2006), proteins from the HSP family are essential biomarkers and can be used to assess cellular responses to pesticide exposure in bees. Due to its sensitivity, this cellular marker has been widely used in ecotoxicology studies to evaluate stress response, particularly in the fat body of various bee species (Balsamo et al., 2023; Farder-Gomes et al., 2024a; Farder-Gomes et al., 2024b).

The fat body is a multifunctional organ found around the organs (perivisceral) and adjacent to the tegument (parietal) in insects, composed of trophocytes and oenocytes (Roma et al., 2010). Among the several functions of the fat body are the storage of organic molecules, synthesis of vitellogenin, hemolymph regulation, immune response, and detoxification (Roma et al., 2010; Arrese and Soulages, 2010; Abdalla and Domingues, 2015). According to the literature, oenocytes are linked to cellular stress response after pesticide exposure (Domingues et al., 2017; Assis et al., 2022; Inoue et al., 2022), supporting the findings observed in this study.

During the larval stage of bees, the fat body exhibits distinct characteristics and is more abundant than in adults due to developmental adaptations specific to this stage (Cruz-Landim, 2009). Despite its abundance, we observed that bees exposed to the highest concentration of picoxystrobin exhibited effects on HSP70 in newly emerged bees. This may suggest that the fungicide remained bioavailable throughout development, leading to a late cellular stress response in this parameter. Similar late effects have also been reported for other fungicides (Tadei et al., 2019; Domingues et al., 2021).

Based on the findings discussed, this research may support future risk assessment programs for bees concerning fungicides, which have received less attention compared to insecticides and herbicides. However, it is important to highlight that this study was conducted under laboratory conditions, which might not take field conditions into account. Future research should look at long-term effects and test these findings in field settings to ensure their applicability in natural environments.

CONCLUSIONS

Considering that the biological parameters of Africanized honey bee larvae were impacted by a single exposure to the highest concentration of fungicide picoxystrobin and based on the knowledge gap in the research field, studies like this reinforce the relevance of intensifying efforts to develop protective actions against larval exposure to fungicides.

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Škodljivi učinki enkratne izpostavljenosti ličink afrikanizirane čebele (*Apis mellifera)* fungicidu pikoksistrobin

IZVLEČEK

Uporaba pesticidov ostaja problem v kmetijstvu, saj onesnažuje naravne ekosisteme in vpliva na čebele. Fungicidi se pogosto uporabljajo po vsem svetu, medonosne čebele pa lahko v svojo čebeljo družino prinesejo kontaminiran cvetni prah in nektar ki vpliva na razvoj ličink. Študije o izpostavljenosti ličink fungicidom so še redke. Zato je bilo to delo namenjeno oceni učinkov izpostavljenosti ličink fungicidu pikoksistrobin na biološke parametre in celični stres v maščobnem telesu. Ličinke so bile četrti dan (D4) enkrat izpostavljene pikoksistrobinu pri koncentracijah 5 ng a.i./µL (PCX5), 45 ng a.i./µL (PCX45), 135 ng a.i./µL (PCX135) in 400 ng a.i./µL (PCX400). Ocenjeni so bili učinki na umrljivost ličink in bub, ter učinki na stopnjo zabubljenja in izleganja. Poleg tega je bil pri na novo izleženih čebelah ocenjen celični stres v maščobnem telesu. Izpostavljenost PCX400 je povečala smrtnost ličink za 26 % in zmanjšala stopnjo izleganja čebel. Druge koncentracije niso vplivale na umrljivost ličink in bub ali na stopnje zabubljenja in izleganja čebel. Učinek citotoksičnosti je bil ugotovljen v novo izleženih čebelah, tretiranih s PCX400, na kar kaže pozitivni imunski test na HSP70. Enkratna izpostavljenost pikoksistrobinu vpliva na slabši razvoj ličink, povzroči celični stresni odziv in potencialno moti dinamiko razvoja čebelje družine.

Ključne besede: razvoj, medonosna čebela, neciljni organizem, strobilurin, toksičnost

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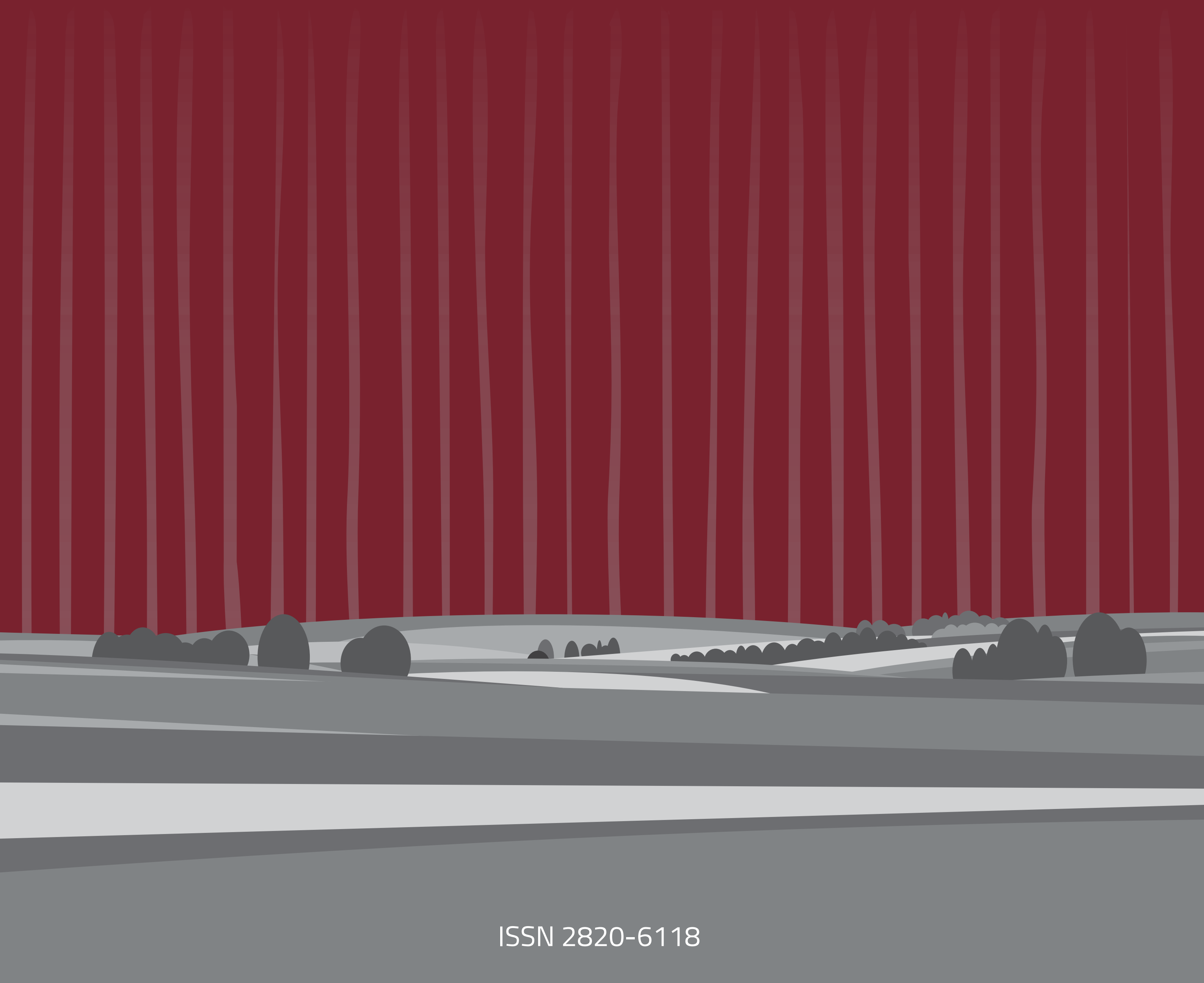

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